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Name of Project: Morphological Differentiation of Colon Carcinoma Cell Lines in Rotating Wall Vessels

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Morphological Differentiation of Colon Carcinoma Cell Lines in Rotating Wall Vessels

Project Objectives: The objectives of this project were to determine whether 1) microgravity permits unique, three-dimensional cultures of neoplastic human colon tissue and 2) this culture interaction produces novel intestinal growth and differentiation factors.

Work Accomplished: The initial phase of this project tested the efficacy of simulated microgravity for the cultivation and differentiation of human colon carcinoma in rotating wall vessels (RWVs) on microcarrier beads. The RWVs simulate microgravity by randomizing the gravity vector in an aqueous medium under a low shear stress environment in unit gravity. This simulation achieves approximately a one-fifth *g* environment that allows cells to "float" and form three-dimensional relationships with less shear stress than in other stirred aqueous medium bioreactors (1). In the second phase of this project we assessed the ability of human colon carcinoma lines to adhere to various substrates because adhesion is the first event that must occur for cells to create three-dimensional masses. Finally, we tested growth factor production in the last phase of this project.

1.) GROWTH IN RWVs

HT-29 and HT-29KM were grown in non-adherent petri dishes and in the RWV with and without Cytodex 3 microcarriers. When HT-29 or HT-29KM were plated in non-adherent Petri dishes and in the RWVs without microcarrier beads, clumps of undifferentiated cells aggregated over 96 hr, but did not proliferate significantly. When HT-29 and HT-29KM cells were cultured in non-adherent petri dishes on microcarrier beads, the tumor cells attached to the beads and formed undifferentiated masses of cells which gradually grew on the beads but did not reach a concentration greater than 1×10^6 cells/ml. However, when HT-29 or HT-29KM was cultured in the RWV on microcarrier beads, the tumor cells achieved concentrations of approximately $2 - 4 \times 10^6$ cells/ml (Appendix 1, 2) and grew as undifferentiated aggregates between beads (Appendix 1, 2). The population doubling time for HT-29 was increased from 24 hr in monolayer cultures to nearly 40 hr in the RWV and the doubling time for HT-29KM was extended from 36 to 61 hr. Diameters of cell aggregates at the termination of the experiments were approximately 0.3-0.5 centimeters with minimal necrosis. When colonic fibroblasts were cultured in the RWVs, they covered and rafted the beads together (Appendix 1, 2). The fibroblasts grew slowly and only attained 0.5×10^6 cells/ml at confluence (Appendix 1, 2). When the histology of each cell type was examined, the fibroblasts appeared similar to connective tissue that appears *in vivo* (Appendix 1, 1). Both HT-29 and HT-29KM monocultures in the RWVs were undifferentiated masses of cells without the appearance of any signet ring cells (Appendix 1, 2).

The tumor cell lines were then cocultivated with normal human colonic fibroblasts. Both of the tumor lines attained higher cell densities when cocultivated with normal human colon fibroblasts than when cultured alone. The HT-29/Fibro co-culture reproducibly attained a concentration of $8.0 - 9.3 \times 10^6$ cells/ml (Appendix 1, 1) while the HT-29KM/Fibro co-culture reached 5.5×10^6 cells/ml (Appendix 1, 2).

More importantly, the co-cultures exhibited several characteristics of morphological differentiation. In the HT-29/Fibro co-cultures, tumor cells grew over fibroblast-covered beads that contained extracellular matrix components produced by the fibroblasts and formed polypoid structures (Appendix 1, 2). HT-29 cells in the cocultures with colonic fibroblasts did not produce glands or mucin-filled signet ring cells in the RWV (Appendix 1, 2). However, HT-29 cells implanted into athymic nude mice also did not differentiate (Appendix 1, 2). Thus, our line of HT-29 exhibited organization and polarity because it grew over fibroblasts that remained closely attached to the microcarrier bead surface and it displayed the same morphology (poor

differentiation) *in vivo* in nude mouse xenografts that it displayed *in vitro* in cocultures in the RWV.

In contrast, the HT-29KM/Fibro co-cultures exhibited morphological differentiation in addition to organization and polarity. Like the HT-29/Fibro co-cultures, HT-29KM cells grew as polyps over fibroblasts on microcarrier beads (Appendix 1, 2). However, unlike HT-29, HT-29KM formed glandular structures that appeared as small "lattice" structures on scanning electron microscopy (SEM) (Appendix 1, 2). These do not appear to be artifacts caused by microcarrier bead impressions because HT-29KM cells formed glands surrounded by fibroblasts in the HT-29KM/Fibro co-cultures (Appendix 1, 2). This looks very similar to a moderately differentiated colon carcinoma that grows in a patient. In addition, signet ring cells were identified in the HT-29KM/Fibro co-cultures that were not present in either HT-29KM monocultures or in HT-29/Fibro co-cultures (Appendix 1, 2). When HT-29KM was implanted in nude mice, it formed a moderately differentiated carcinoma with both gland formation and the production of signet ring cells (Appendix 1, 2). The appearance of the HT-29KM/Fibro co-cultures is similar to the appearance of HT-29KM cells grown in nude mice. Thus, HT-29KM demonstrates epithelial polarity and organization and produces glands and signet ring cells when cultured in a three-dimensional environment with mesenchymal cells *in vitro* in the RWV or *in vivo* in the nude mouse. These morphological data suggest that the RWVs support the development of tissues that are remarkably similar to structures that are formed *in vivo*.

An important question is whether this differentiation induced by fibroblasts is due to the low shear stress environment of the RWV or whether this will also occur in conventional unit gravity culture. The criteria for differentiation in these monolayer cultures were an increase in desmosomes (the intercellular connections that are the major bonds linking differentiated epithelial cells together), production of mucin by the appearance of signet ring cells, or the development of tumor glands. None of these markers of differentiation were increased in HT-29 or HT-29KM cultures by placing the cells in monolayer culture and exposing them to such differentiation agents as sodium butyrate or dimethyl sulfoxide (data not shown). Cultivation of either HT-29 or HT-29KM with human fibroblasts in monolayer cultures did not induce mucin expression but did cause the colon carcinoma cells to grow as clusters surrounded by fibroblasts (Appendix 1, 2). However, induction of signet ring cells did not occur in HT-29KM cells (Appendix 1, 2).

The stimulus that triggers the morphological differentiation of HT-29KM or prolongs the cell cycle in both carcinoma cell lines cocultured with human colon fibroblasts is not known. However, the metabolism of glucose may provide a clue. Human fibroblasts, HT-29, and HT-29KM cultured as monocultures in the RWV under low shear stress conditions (3 - 5 mm bead aggregates with an estimated 0.5 - 2 dynes cm⁻² surface shear stress) consume significantly less glucose than in monolayer cultures (Table 1, below). When either HT-29 (Figure 1A, below) or HT-29KM (Figure 1B, below) is cocultured in the RWV with fibroblasts, the GUR decreases as the coculture increases in cell number. While this may reflect the relative proportions of tumor to normal cells present at any time in the culture, it may also reflect a change in the consumption of glucose by carcinoma cells because the approximate ratio of tumor cells to normal fibroblasts is at least 9:1 beginning at day 4. This mechanism of decreased glucose consumption in the RWV has not been pursued further because we do not have the gene probes to investigate glucose transport (to evaluate glucose uptake effects) or metabolism. Nonetheless, the observation that glucose consumption is decreased as the RWV cocultures of HT-29 and HT-29KM grow and differentiate supports an association between glycolysis and morphological differentiation- an observation supported by others (3).

We have cultured other human colorectal carcinomas as monocultures in the RWV to determine their growth characteristics. A moderately differentiated carcinoma (CCL 188, Figure 2) and two poorly differentiated carcinomas (KM-12c, Figure 3, and MIP-101, Figure 4) grow well as monocultures in the RWV but only attain maximum concentrations of 1 - 3 x 10⁶ cells/mL. The GUR's of these carcinoma cultures increase as the culture progresses (Figures 2 - 4), but decrease when calculated on a per cell basis (data not shown). When MIP-101 cells were cultured with BALB/c 3T3 embryonic fibroblasts in the RWV, the culture failed to proliferate (Figure 5). The

3T3 cells covered the microcarrier beads but did not support the growth of the MIP-101 cells. In fact, the MIP-101 cells detached from the microcarrier beads and remained in suspension but did not proliferate. Since MIP-101 cells were explanted from the ascites of a patient with widely disseminated disease (4), our interpretation was that the 3T3 cells stimulated the MIP-101 cells to behave the way they did in the original host and to float in suspension in loose aggregates. We used 3T3 cells because the earlier studies with HT-29 and HT-29KM suggested that mouse stromal cells stimulate morphological changes in human carcinoma cells to the same degree as human colonic fibroblasts do. 3T3 cells represent an embryonic fibroblast that is pluripotent and may be able to support a variety of cell types. Nonetheless, we thought that the MIP-101 co-cultures would form tissue masses and were disappointed that they did not. We attempted to coculture HT-29KM with 3T3 cells but had trouble initiating the cultures. We were not able to determine why this was a problem. We also were not able to establish primary cultures of normal human colonic fibroblasts because we could not get sufficient normal colon mucosa specimens from the pathologists at the New England Deaconess Hospital. Thus, we next studied the effect of simulated microgravity on the ability of neoplastic epithelial cells to adhere to various substrates.

2. CELL ADHESION STUDIES

Production of complex tissues requires that individual cells recognize and adhere to other cells and to extracellular matrix and basement membrane molecules. Microgravity may be useful in engineering new tissues *in vitro* because the lack of sedimentation may permit the formation of tissues with high fidelity their *in vivo* counterparts. However, microgravity must not alter the ability of cells to recognize and adhere to extracellular matrices and intercellular ligands if it is to be used to construct tissues. The purpose of this study was to test the hypothesis that microgravity does not effect cell adhesion. This was tested by culturing human colon carcinoma cells for short (6-7 day cultures) or long (23 days) periods in the RWV and then assessing cell adhesion to the basement membrane proteins laminin and type IV collagen as well as to the intercellular adhesion molecule carcinoembryonic antigen (CEA). The results indicate that microgravity does not decrease the ability of neoplastic epithelial cells to adhere to basement membrane proteins even after a long period of culture in the RWV (Appendix 2, 5). Interestingly, the nonspecific adherence to bovine serum albumin and to the polystyrene surface of the microtiter wells in which the assay was performed was increased (Appendix 2, 5), although the significance of this is not clear. Although the ability of all the cell lines tested to adhere to standard ligands was not impaired functionally, the expression of one adhesion receptor, CD44, was significantly decreased on MIP-101 cells cultured for 23 days. These results suggest that cells will recognize and attach to standard ligands that are important for tissue construction *in vivo* when they are cultured in microgravity. However, there may be changes in the types of receptors that cells use to adhere to substrates and to each other.

3. GROWTH FACTOR PRODUCTION

Microgravity may permit cultivation of mammalian cells in a three dimensional aqueous matrix that creates tissue *in vitro* that is functionally and structurally similar to tissue formed *in vivo*. The last objective of this project was to determine whether growth factor production by human colonic cells is enhanced in simulated microgravity compared to standard tissue culture conditions and to tissues *in vivo* in the intact animal. We first assayed growth factor production in an *in vivo* assay in mice where conditioned medium was placed within the bowel wall and its effects on normal epithelium was analyzed morphologically. This assay had two drawbacks: first, human factors may not affect mouse colonocytes and second, the assay was technician-dependent since not all technicians could inject 20 μ l quantities of liquid into the submucosa of a 20 - 25 gm mouse. Thus, considerable effort was expended on developing an *in vitro* assay to measure factor production. Finally, the production of defined growth factors was evaluated to provide a background for these studies.

A. IN VIVO GROWTH FACTOR ASSAY

If simulated microgravity conditions induced growth factor production similar to that observed in vivo and not produced in other culture systems, then actual microgravity may be even more advantageous for the production of tissues in culture. Human colonic carcinoma cell lines HT-29 and HT-29KM were co-cultured with human fibroblasts to form tissue masses in the RWV that are morphologically similar to the carcinoma grown as metastases in vivo in athymic nude mice (Appendix 1, 2). We had previously shown that freshly isolated human colorectal adenocarcinomas implanted in the wall of the large bowel of athymic nude mice release soluble factors that stimulate selective proliferation of goblet cells in the colonic mucosa overlying the tumor implants (6). Goblet cells contain mucin that lubricates and binds fecal contents for excretion and are easily distinguished from the other epithelial cells that line the crypts of Lieberkuhn which is the mucosal lining of the large bowel. Medium was conditioned by HT-29 cells grown on microcarrier beads in either the RWV or in conventional roller bottles. The conditioned media were then compared in a bioassay to fresh medium and to purified growth factors for their effects upon normal murine colonocytes by incorporating media in liposomes that were injected into the submucosa of normal BALB/c mice. After 5 days, the mice were sacrificed and the bowel harvested and examined histologically. Colonic epithelial cells in the crypts of Lieberkuhn were enumerated in a coded fashion for the total number of cells, the number of goblet cells, and the height of each crypt. An increase in the number of cells within each crypt is hyperplasia. An increase in the height of the crypt without an increase in the number of cells per crypt is an enlargement of each cell within the crypt and is termed hypertrophy.

Fresh medium did not stimulate hyperplasia, hypertrophy or changes in the relative proportion of goblet cells compared to saline controls. In contrast, India ink caused acute inflammation and increased crypt height, cell number, and goblet cells per crypt without increasing the percentage of goblet cells (Appendix 3, 7). Media from cultures of HT-29 or HT-29KM in either roller bottles or the RWV increased the height of crypts, the number of cells per crypt, and the absolute number of goblet cells per crypt compared to saline controls (Appendix 3, 7). However, media from RWV cultures selectively increased the percentage of goblet cells whereas media from roller bottle cultures only increased the total number of crypt cells (Appendix 3, 7). These effects of conditioned media were due to factors released by carcinoma cells because media from human fibroblast cell cultures in the RWV did not produce any effect on crypt height or cell number. Although the factor(s) responsible for the selective expansion of goblet cells have not yet been isolated, purified growth factors (epidermal growth factor - EGF, basic fibroblast growth factor - bFGF, and gastrin) were hyperplastic or hypertrophic but did not increase the number of goblet cells (Appendix 3, 7).

Cultivation of established human colon carcinoma cells in the simulated microgravity of the RWV produced an activity that selectively expanded goblet cells in normal mouse bowel epithelium. Similar cultures in roller bottles did not achieve the same selective expansion of goblet cells, although there was a trophic effect on the bowel mucosa. While the goblet cell stimulating activity has not yet been isolated, it is possible that a novel intestinal peptide is responsible for this activity. It is also possible that previously described factors acting either alone or in concert may have similar effects on selectively expanding the different colonocytes. However, purified factors (EGF, bFGF, and gastrin) that are trophic for colonic mucosa did not have any specific effect on goblet cells when added in pharmacological doses. Since freshly isolated human colon carcinomas have the same effect on goblet cells as the RWV HT-29 colon cell line cultures did, the simulated microgravity of the RWV may provide a better three-dimensional environment for isolating novel factors than conventional batch culture systems. In addition, the ability of human colon carcinomas to selectively stimulate the population of goblet cells suggests that there is a factor that we tentatively termed goblet cell differentiating factor (GCDF).

B. EGF, bFGF, TGF β 1 PRODUCTION IN THE RWV BY HUMAN COLON CARCINOMAS

The amounts of EGF, bFGF, and TGF β 1 were measured in the conditioned medium from monocultures of CCL 188, KM-12c, and MIP-101 to better define growth factor production in the RWV. Commercially available monospecific enzyme immunoassay (EIA) kits were used to measure the amounts in picogram quantities. EGF and bFGF were not detectable in any conditioned medium from RWV or monolayer cultures of any of these human colon carcinoma cell lines or cultures of HT-29KM (data not shown). However, interesting associations were noted between TGF β 1 production and the proliferation of colon carcinoma lines. Friedman and her colleagues have data that suggest that poorly differentiated carcinomas may use TGF β 1 as a growth stimulatory factor while the proliferation of moderately differentiated carcinomas is inhibited by TGF β 1 (8). Interestingly, monocultures of human colon carcinoma cell lines in the RWV are consistent with these findings. CCL 188 is a moderately differentiated carcinoma whose production of TGF β 1 increases as the viability and cell number of the RWV culture decreases (Appendix 4, Figure 2). In contrast, the production of TGF β 1 decreases as the poorly differentiated carcinomas KM-12c (Appendix 4, Figure 3) and MIP-101 (Appendix 4, Figure 4) reach their growth plateaus. We did not measure TGF α because we did not have a kit for this factor. However, the complete absence of detectable EGF suggests that the amounts of TGF α are likely to be small. Thus, while TGF α must be measured, nonetheless the association among differentiation of human colon carcinoma, proliferation, and production of the autocrine factor TGF β 1 supports the findings of others (8) in conventional systems.

C. IN VITRO GCDF ASSAY

Our original finding that cocultures of HT-29KM with fibroblasts in the RWV induced the appearance of mucin-filled signet ring cells with concomitant production of GCDF in the conditioned medium prompted us to attempt to develop an in vitro assay to isolate and purify GCDF and other potentially novel factors. Considerable effort was expended on this part of the project but with little to show because the in vitro results were hard to reproduce. Our strategy was to perform a cell-based EIA for specific mucin epitopes. The two epitopes were defined by monoclonal antibodies B72.3, which is directed to the Tn and sialylated Tn antigen present on a wide variety of human colon and other carcinomas (9), and DF3, which is directed to a peptide within the tandem repeats of small mucin (10). Both of these monoclonal antibodies measure different sialomucins that are expressed by human colon carcinoma lines and are murine IgG1 immunoglobulins. As a result, we used MOPC 31c, an IgG1 murine myeloma protein, as a control. When the cell lines grown in monolayer culture were surveyed for mucin production, MIP-101 displayed essentially no DF3 or B72.3 epitope while all other human colon carcinoma lines produced some detectable amounts of each (data not shown). When conditioned medium from HT-29KM cultures was incubated for 5 days with MIP-101 cells in 96-well microtiter plates in varying concentrations with fresh RPMI 1640 and 10% FCS, there was a significant increase in the amount of B72.3, but not DF3, epitope expressed by the MIP-101 cells (Appendix 4, Figure 5). Attempts to identify the size of the putative factor(s) that enhance the expression of this epitope were made by subjecting serum-free preparations of HT-29KM conditioned medium from RWV cultures to molecular sieving chromatography on Sephadex G75 columns. No consistent results were obtained. This suggests that either GCDF is an artifact or the factor is too unstable to be identified in the type of assay that we studied.

Although we were not successful in isolating GCDF, we did determine that EGF and TGF β 1 may interact to increase the expression of the epitope B72.3 on MIP-101 cells. This is suggested by the finding that increasing the amount of EGF in the medium increases the specific binding of B72.3 to MIP-101 cells while TGF- β 1 does not effect the amount of B72.3 epitope expressed (Appendix 4, Figure 6A). The addition of antibody to TGF β 1 to the medium was synergistic with EGF in increasing the amount of B72.3 epitope on MIP-101 cells. Thus, we have

shown that EGF and TGF β 1 may alter the amount of Tn and sialyl Tn expressed by MIP-101 cells.

CONCLUSION

In summary, this project has shown that a number of human colon carcinoma cell lines may be successfully cultured in the RWV. In addition, factors may be produced in the spent medium that are trophic to colonic epithelium and may stimulate differentiation. Unfortunately, we were not able to create an in vitro assay to purify GCDF. However, we did demonstrate that TGF β 1 and EGF have contrasting effects on the expression of sialomucins by human colon carcinomas.

Publication List Resulting From This Project:

- 1.) Goodwin TJ, Jessup JM, Wolf DA. Morphological differentiation of HT-29 and HT-29KM in rotating wall vessels. *In Vitro Cell. Develop. Biol.* 28A(1):47-60, 1992.
- 2.) Jessup JM, Brown K, Ishii S, Ford R, Goodwin TJ, and Spaulding G. Simulated microgravity does not alter epithelial cell adhesion to matrix and other molecules. *Adv. Space Res.* 14:(8)71-(8)76, 1994.
- 3.) Jessup JM, Goodwin TJ, Spaulding G. Prospects for use of microgravity-based bioreactors to study three-dimensional host-tumor interactions in human neoplasia. *J. Cell. Biochem.* 51(3):290-300, 1993.
- 4.) J.M. Jessup and T.J. Goodwin. Simulated microgravity improves production of colonic growth factors. Technical paper AIAA-93-4098, 1993.
- 5.) J.M. Jessup and T.J. Goodwin. Simulated microgravity improves production of colonic growth factors. Presented at AIAA meeting in September, 1993 in Huntsville, AL.
- 6.) J.M. Jessup, R. Ford, G. Spaulding, and T.J. Goodwin. Use of microgravity emulation to test the function of three-dimensional human colon carcinoma cell cultures. Presented at COSPAR meeting in August 1992 in Washington, D.C.
- 7.) J.M. Jessup, R. Ford, T.J. Goodwin, G. Spaulding. Effect of simulated microgravity on human epithelial cell association and recognition. Presented at the national meeting of the Aerospace Medical Association in Miami, Florida in May 1992.
- 8.) J.M. Jessup, T.J. Goodwin, D.A. Wolf, G. Spaulding. Growth of human colorectal carcinoma cells in nasa rotating wall vessel (RWV) produces high cell yields with differentiation and reduced glucose consumption. Presented at the UCLA Symposium on Tissue Engineering, Taos, NM, January, 1994.
- 9.) J. M. Jessup, W. Fitzgerald, R. Ford, J. Polanec, and D. Brown. CEA production in human colon cancer. Presented at the national meeting of the Tissue Culture Association, June, 1994.

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- 1.) Jessup JM, Goodwin TJ, Spaulding G. Prospects for use of microgravity-based bioreactors to study three-dimensional host-tumor interactions in human neoplasia. *J. Cell. Biochem.* 51(3):290-300, 1993.

- 2.) Goodwin TJ, Jessup JM, Wolf DA. Morphologic differentiation of colon carcinoma cell lines HT-29 and HT-29KM in rotating-wall vessels. In Vitro Cell. Develop. Biol. 28A(1):47-60, 1992.
- 3.) Wice BM, Trugnan G, Pinto M, Rousset M, Chevalier G, Dussault E, Lacroix B, Zweibaum A. The intracellular accumulation of UDP-N-acetylhexosamines is concomitant with the inability of human colon cancer cells to differentiate. J. Biol. Chem. 260(1):139-46, 1985.
- 4.) Niles RM, Wilhelm SA, Steele GD Jr, Thomas P and Zamcheck N. Isolation and characterization of an undifferentiated human colon carcinoma cell line (MIP-101). Cancer Invest. 5: 545-552, 1987.
- 5.) Jessup JM, Brown K, Ishii S, Ford R, Goodwin TJ, and Spaulding G. Simulated microgravity does not alter epithelial cell adhesion to matrix and other molecules. Adv. Space Res. 14:(8)71-(8)76, 1994.
- 6.) Hostetter RB, Cleary KR, and Jessup JM. Transitional Mucosa: Cause or result of human colon carcinogenesis? Surg Forum 39:168-170, 1988.
- 7.) J.M. Jessup and T.J. Goodwin. Simulated microgravity improves production of colonic growth factors. Technical paper AIAA-93-4098, 1993.
- 8.) Hafez MM, Hsu S, Yan Z, Winawer S, Friedman E. Two roles for transforming growth factor beta 1 in colon enterocytic cell differentiation. Cell Growth & Differentiation. 3(11):753-62, 1992.
- 9.) Irimura T, Matsushita Y, Hoff SD, et al: Ectopic expression of mucins in colorectal cancer metastasis. Semin Cancer Biol. 2:129-39, 1991
- 10.) Andrews CW Jr, Jessup JM, Goldman H, Hayes DF, Kufe DW, O'Hara CJ, Steele GD Jr. Localization of tumor-associated glycoprotein DF3 in normal, inflammatory, and neoplastic lesions of the colon. Cancer 72:3185-90, 1993.

Appendices

- 1.) Goodwin TJ, Jessup JM, Wolf DA. Morphologic differentiation of colon carcinoma cell lines HT-29 and HT-29KM in rotating-wall vessels. In Vitro Cell. Develop. Biol. 28A(1):47-60, 1992.
- 2.) Jessup JM, Brown K, Ishii S, Ford R, Goodwin TJ, and Spaulding G. Simulated microgravity does not alter epithelial cell adhesion to matrix and other molecules. Adv. Space Res. 14:(8)71-(8)76, 1994.
- 3.) J.M. Jessup and T.J. Goodwin. Simulated microgravity improves production of colonic growth factors. Technical paper AIAA-93-4098, 1993.
- 4.) Miscellaneous Data
- 5.) Jessup JM, Goodwin TJ, Spaulding G. Prospects for use of microgravity-based bioreactors to study three-dimensional host-tumor interactions in human neoplasia. J. Cell. Biochem. 51(3):290-300, 1993.
- 6.) Abstracts

**MORPHOLOGICAL DIFFERENTIATION OF COLON CARCINOMA CELL
LINES HT-29 AND HT-29KM IN ROTATING-WALL VESSELS**

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Running Head: Differentiation in rotating-wall vessels

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SUMMARY

A new low shear stress microcarrier culture system has been developed at NASA's Johnson Space Center that permits three-dimensional tissue culture. Two established human colon adenocarcinoma cell lines, HT-29, an undifferentiated, and HT-29KM, a stable, moderately differentiated subline of HT-29, were grown in new tissue culture bioreactors called Rotating-Wall Vessels (RWVs). RWVs are used in conjunction with multicellular cocultivation to develop a unique *in vitro* tissue modeling system. Cells were cultivated on Cytodex-3 microcarrier beads, with and without mixed normal human colonic fibroblasts, which served as the mesenchymal layer. Culture of the tumor lines in the absence of fibroblasts produced spheroid-like growth and minimal differentiation. In contrast, when tumor lines were cocultivated with normal colonic fibroblasts, initial growth was confined to the fibroblast population until the microcarriers were covered. The tumor cells then commenced proliferation at an accelerated rate, organizing themselves into three-dimensional tissue masses that achieved 1.0- to 1.5-cm diameters. The masses displayed glandular structures, apical and internal glandular microvilli, tight intercellular junctions, desmosomes, cellular polarity, sinusoid development, internalized mucin, and structural organization akin to normal colon crypt development. Differentiated samples were subjected to transmission and scanning electron microscopy (TEM, SEM) and histological analysis, revealing embryonic-like mesenchymal cells lining the areas around the growth matrices. Necrosis was minimal throughout the tissue masses. These data suggest that the RWV affords a new model for investigation and isolation of growth, regulatory, and structural processes within neoplastic and normal tissue.

Key Words: rotating-wall vessels; tissue modeling; microcarrier; three-dimensional; differentiation; colon cancer

INTRODUCTION

An important principle of cellular physiology is the interaction of multiple cell types and their association with cellular differentiation. As predicted by embryologists for more than 50 years, cellular differentiation is effected and maintained by complex cellular interactions.(13,36,38) Mechanisms for these cellular interactions involve cell membrane junctions, extracellular matrices (e.g., basement membrane and ground substances), and soluble signals (autocrine, paracrine, and endocrine). In human endometrium, isolated epithelium is not responsive to estrogen in spite of the presence of estrogen receptors.(20) presumably due to a lack of other tissues necessary for the proliferative response observed *in vivo*. Although cellular differentiation is genetically based, the process is also influenced by the cellular environment, particularly the three-dimensional spatial relationship of cells to each other and to extracellular matrices and factors.(5,8,9,16)

For long-term culture, the best results are obtained in epithelial cells cultured in association with a fibroblast or endothelial "feeder" layer. In hepatocytes, for example, this configuration improves both culture longevity and tissue-specific functions such as hydrocortisone-inducible tyrosine aminotransferase. Coculture of rat hepatocytes with endothelium and Kupffer cells resulted in greater retention of the ultrastructural markers that distinguish these cell types. Furthermore, conditioned medium from a "feeder" cell line did not replace the need to cocultivate with an additional cell type.(14,29)

Present *in vitro* culture technology does not permit reproducible cultures of normal or neoplastic colonic epithelium in large-scale, three-dimensional configurations.(6) As a consequence, the factors that control proliferation and differentiation in the gastrointestinal tract remain largely unknown.(1,2,3,7,23,28) Culture of intestinal epithelial cells by standard techniques has limited success. One of the first techniques, organ culture, in which slices of tissue were placed in culture medium, permitted only short-term analysis of crypt population

dynamics. Long-term cultures were difficult to achieve (longer than 7 weeks), since crypt cells are unable to survive standard culture regimens.(32) Unfortunately, two-dimensional organ culture does not support the assembly of the stroma and its contribution to the growth of epithelial cells. As a result, investigators resorted to the culture of normal gut-lining epithelial cells in monolayer cultures. While short-term cultures are possible, long-term growth has required transformation with a tumor promoter, oncogenic virus, or culture in highly defined, exotic media often resulting in the loss of cellular organization and function.(22) Short-term cultures of normal colon cells developed an oriented monolayer with an absorptive apical surface, junctional complexes, and dome formation. In addition, overlays of basement membrane preparations aided in the differentiation of some cultures of neoplastic colonic epithelium.(4,17,18,19) The culture of primary human large bowel neoplastic cells, which have less stringent growth requirements than normal colonic epithelium, has a low success rate. Only 18% to 29% of adenocarcinomas of the colon or rectum can be established *in vitro*. In comparison, 62% of colorectal carcinomas may be established as xenografts in athymic nude mice.(15) While not all human carcinomas propagate in immunocompromised mice, more carcinomas grow in nude mice than in current tissue culture systems. Although animal models are useful for certain studies, many biochemical and molecular studies require that cells be grown *in vitro*. Due to the lack of a successful long-term technology to culture gut epithelium, a new technology developed at NASA's Johnson Space Center is now being used to construct a large-scale, three-dimensional *in vitro* tissue culture model which compares favorably with the nude mouse model. Briefly, RWVs are horizontally rotating cylindrical tissue culture vessels which provide controlled supplies of oxygen and nutrients, with minimal turbulence and extremely low shear.(39) These vessels suspend cells and microcarriers homogeneously in a nutrient-rich environment which allows the three-dimensional assembly of cells to tissue.

MATERIALS AND METHODS

Cell lines. Two colon adenocarcinoma cell lines were used: HT-29, an undifferentiated adenocarcinoma originally isolated by J. Fogh at the Walker Laboratory, Rye, NY,(12) and HT-29KM, a moderately differentiated subline of HT-29 isolated by passage through athymic BALB/c nude mice. Karyotypic analysis of the two lines demonstrates that HT-29 has a hyperdiploid complement of chromosomes (Figure 1A) while HT-29KM is near diploid but contains several marker chromosomes of HT-29 (Figure 1B). Further, HT-29 grows as an undifferentiated tumor xenograft that lacks gland formation (Figure 9A) while HT-29KM is a moderately differentiated xenograft that forms glands and mucin-containing signet ring cells (Figure 9C). Normal adult colon fibroblasts were established from primary cultures of the normal colons of organ donors.(15) Cellular purity was established by vimentin positive, AE1/AE3 and Factor VIII antibody negative immunocytochemistry. The normal fibroblast cell line CCD-112CoN and our HT-29 cultures were obtained from the American Type Culture Collection (Rockville, MD).

Monolayer culture. All cells were grown in monolayer culture in a Forma Stericult 100 humidified CO₂ incubator. The cultures were maintained in a 95% air 5% CO₂ constant atmosphere at a temperature of 37°C. Growth medium for all cultures was Minimal Essential Medium Alpha (MEM Alpha) with 100 mg/dl glucose (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT); 1% nonessential amino acids, 1% MEM vitamins, 2 g/l HEPES, and 1% L-Glutamine, 100 units Penicillin, 100 µg streptomycin, and 2.5 µg/ml Fungizone (GIBCO); and 2.5 µg/ml insulin, 2.5 µg/ml human transferrin, and 2.5 ng/ml sodium selenite (Sigma Chemical Co., St. Louis, MO).

Cells were passaged as required by enzymatic dissociation with a solution of 0.1% Trypsin, 0.1% EDTA, for 15 minutes. After incubation with the appropriate enzymes, the cells were centrifuged at 800 g for 10 minutes in Corning Conical 15-ml centrifuge tubes. The cells

were then resuspended in fresh medium and diluted into Corning T-75 flasks with 25 ml of fresh growth medium.

Monolayer controls for Rotating-Wall Vessel experiments. Samples from the cellular inoculum for each RWV experiment (2×10^5 cells/ml) were placed in Corning T-75 flasks to serve as growth controls. For cocultivation experiments the two cell types were mixed in a predetermined ratio and placed in culture flasks and in Dispo nonadherent petri dishes (100 x 15 mm) (Lab-Tek Division of Miles Laboratories, Naperville, IL 60540) with microcarrier beads. The progress of control cultures was monitored and recorded photographically on a Nikon Diaphot Inverted Microscope equipped with Hoffman Modulation Optics.

Rotating-Wall Vessel cultures. The Rotating-Wall Vessel is a horizontally rotated, zero headspace, center oxygenation, transparent culture vessel. Cells to be cultured in the RWV were grown initially in T-flasks as described above in preparation for seeding into the vessels. The initial inoculum for each experiment was 2×10^5 cells/ml, with 5 mg/ml Cytodex-3 microcarriers (Pharmacia, Piscataway, NJ) which resulted in a starting concentration of 10 cells per bead. Cytodex-3 microcarriers are type 1, collagen coated dextran beads of 175 microns in diameter. The concentration of beads was maintained at 5 mg/ml. Cocultivation experiments used 10% tumor cells and 90% fibroblasts as the starting inoculum.

Tumor cells and fibroblasts were removed from T-75 flasks by enzymatic digestion, washed once with calcium- and magnesium-free PBS, and assayed for viability by Trypan Blue dye exclusion (GIBCO). Cell types were mixed in the proper ratio and placed in growth medium on ice prior to inoculation. After inoculating the RWV, the cultures were allowed to grow for 48 hours before the medium was changed. Thereafter, the medium was changed every 20 to 24 hours; as the metabolic requirements of the cultures increased, fresh

medium was supplemented with an additional 100 to 200 mg/dl of glucose. The growth curves for each experiment were reported as average number of cells per ml vs. incubation time.

Histological analysis. Samples from RWV cultures were taken at multiple time points throughout the course of the experiments for histological analysis. Two basic stains were performed on the 20, 10, and 6 μ sample sections, hematoxylin and eosin (H&E) and mucicarmine staining. After removal from the reactor vessels, samples were washed once with calcium- and magnesium-free PBS. The samples were suspended in a buffer containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.4 and analyzed by histology and electron microscopy.(21)

Mucicarmine staining was performed according to the procedure of Sheehan, et al.(33) Tissues were fixed as previously stated. The samples were embedded in paraffin, blocked, and cut in multiple thicknesses as stated above. Sections were then mounted on slides and deparaffinized. The preparations were stained with Weigert's Iron Hematoxylin Working Solution for 5 minutes, then rinsed with running tap water for 5 minutes and placed in Mucicarmine Working Solution for 30 minutes at room temperature. Slides were then rinsed with deionized water, stained with tartrazine solution for 1 to 5 seconds, then rinsed again with deionized water. The slides were dehydrated with xylene and mounted for analysis.

Scanning and transmission electron microscopy. Samples from the RWV cultures were taken for SEM at the same times as those taken for histological analysis. Samples were fixed as described above, then rinsed for 5 minutes with cacodylate buffer three times and postfixed with 1% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA) in cacodylate buffer for 1 hour. Samples were then rinsed for 5 minutes with distilled water three times and then treated for 10 minutes with Millipore (Millipore Corp., Bedford, MA) (0.2 μ) filtered saturated solution of thiocarbohydrazide (Electron Microscopy Sciences), then washed

for 5 minutes with distilled water five times and fixed with 1% buffered osmium tetroxide for 10 minutes. This last step was necessary to prevent the beads from collapsing. Samples were then rinsed with distilled water three times and dehydrated with increasing concentrations of ethanol followed by three changes in absolute methanol, and divided for TEM and SEM.

Samples for SEM were transferred to 1, 1, 1, 3, 3, 3-hexamethyldisilazane (HMDS) (Electron Microscopy Sciences) where they were allowed to soak for 10 minutes. Samples were then drained and allowed to air dry overnight. Dried samples were sprinkled with a thin layer of silver paint on a specimen stub. The paint was allowed to dry and the samples were coated by vacuum evaporation with platinum-palladium alloy. The samples were then examined in the AMRAY 1000 SEM at an accelerating voltage of 5 to 10 kV.

Samples for TEM were fixed as were all other samples in 3% gluteraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. They were washed in 0.1 M cacodylate buffer (3 x 10 minutes), post fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer and washed in 0.1 M cacodylate buffer (3 x 10 minutes). Specimens were dehydrated in an ascending series of acetone (30, 50, 70, 95 and 2 x 100%, 5 minutes each), transferred to propylene oxide (2 x 10 minutes) and infiltrated in Spurr low viscosity embedding medium. The infiltration schedule consisted of increasing ratios of Spurr resin: propylene oxide (1:1, 1 hour; 2:1, 2 hours; and pure resin, 4 hours). Individual pieces, approximately 2-4 mm³, were transferred to silicone flat embedding molds containing fresh Spurr resin, and the resin was polymerized at 60°C for 24 hours. Thin sections (60-80 nm) were cut using a diamond knife on a Reichert Ultracut E ultramicrotome. The sections were collected onto naked 300 hexagonal mesh grids and were stained with saturated aqueous uranyl acetate and Reynolds lead citrate. The sections were examined and photographed using a Philips CM12 TEM operating at 80 kV.

Quantitation of morphological structure. The number of polyps and glands and the size of tissue masses were quantitated from 5-ml samples extracted from the RWV at the conclusion of each coculture experiment. Tissue masses were placed in 35-mm petri dishes (Limbco) with 2-mm grids and quantitated with an inverted Nikon Diaphot Microscope equipped with phase contrast optics.

RESULTS

Cultures in nonadherent petri dishes. HT-29, HT-29KM, and primary normal colon fibroblasts were cultured in nonadherent petri dishes with and without microcarrier beads. When each of the three cell types was plated without microcarrier beads, clumps of undifferentiated cells aggregated and died over a 96-hour period. Low cell yields were obtained when HT-29 and HT-29KM were cultured separately, each with microcarrier beads.(11) Tumor cells attached to the beads and formed undifferentiated masses of cells eventually achieving only 1.0×10^6 cells/ml. When primary normal colon fibroblasts were cultured with microcarrier beads, the cells covered the beads and rafted some beads together, finally becoming contact-inhibited, but left many bare beads. These cultures (Table 1) only reached a cell density of 0.3×10^6 cells/ml.

Monocultures in Rotating-Wall Vessels. When HT-29 or HT-29KM was cultured in RWVs on microcarrier beads, the starting inoculum was 2.0×10^5 cells/ml, and the tumor cells achieved culture-densities of approximately 2 to 4×10^6 cells/ml (Figure 2A) and grew as undifferentiated aggregates between beads (Figures 3C, 3D, 3E, and 3F). The population doubling time for HT-29 increased from 24 hours in monolayer cultures to nearly 40 hours in the RWV; the doubling time for HT-29KM was extended from 36 to 61 hours. Cell aggregates reached approximately 0.3 to 0.5-cm diameters with minimal necrosis. When colonic fibroblasts were cultured in the RWVs, they covered and rafted the beads (Figures 3A, and 3B). The fibroblasts grew slowly and became contact-inhibited, attaining a concentration of only 0.5×10^6 cells/ml and were maintained at confluence for 5 days (Figure 2A). When each cell type was examined for evidence of differentiation, the fibroblasts appeared similar to the connective tissue that appears *in vivo* (Figures 4A, and 5A). HT-29 appeared to form undifferentiated masses of cells (Figures 3C, 4B, 5B, 6A, 6B, and 6C), and HT-29KM also appeared largely undifferentiated with occasional intracellular glands but no signet ring cells, cellular polarity, columnar development, or apical microvilli (Figures 3E, 4D, 5D, 6D, 6E, and 6F).

Cocultivation in Rotating-Wall Vessels. HT-29 and HT-29KM were also cocultivated with normal human colonic fibroblasts and the starting inoculum for each coculture was 2.0×10^5 cells/ml consisting of a 9:1 ratio of fibroblasts to tumor cells.(18,30) Each tumor line attained higher cell densities when cocultured than when cultured alone: the HT-29/Fibro coculture attained 8.0 to 9.3×10^6 cells/ml (Figure 2B), and the HT-29KM/Fibro coculture reached 5.5×10^6 cells/ml (Figure 2C).

More importantly, the cocultures exhibited several characteristics of morphological differentiation.(6) In the HT-29/Fibro cocultures, tumor cells grew over fibroblast-covered beads and formed polypoid structures (Figures 4C and 5C). However, these cocultures did not produce mucus-containing signet ring cells (Figures 4C, 5C, 7E, 7F, and 9B). In contrast, the HT-29KM/Fibro cocultures exhibited enhanced morphological differentiation and organization of polarity.(27) Similar to HT-29/Fibro cocultures, HT-29KM cells grew as polyps over fibroblasts on microcarrier beads. However, unlike HT-29, HT-29KM formed glandular "lattice" structures as visualized with SEM (Figures 8C and 8D) and histology. These cultures also formed organized epithelium (Figures 10A, 10B, and 10C) with apical microvilli, columnar epithelium (Figure 10D), large amounts of extracellular matrix material (Figure 10E) and interglandular structures (Figure 10F) which contained mucinous material as verified by mucicarmine stains (Figure 5E). It is unlikely that these structures are artifacts caused by microcarrier bead impressions, because these glands were surrounded by fibroblasts (Figure 8F), and looked very similar to a moderately differentiated colon carcinoma *in vivo*. Finally, signet ring cells were identified(24,26) in the HT-29KM/Fibro cocultures that were not present in either HT-29KM alone or in HT-29/Fibro cocultures (Figures 4E, 5E, 7E, and 9D). The appearance of signet ring cells and glands in the HT-29KM/Fibro cocultures is similar to the appearance of HT-29KM grown in nude mice.

The number of polyps, glands, and tissue masses of different sizes was quantitated in 5-ml aliquots of medium harvested from RWVs that were plated in 35-mm petri dishes with a 2-mm grid. HT-29/Fibro cocultures averaged 18 polyps/5 ml 15 to 19 days after initiation of culture (Figure 11A). HT-29KM/Fibro cocultures had slightly fewer polyps, but formed an average of 35 glands/5 ml of medium harvested (Figure 11A). HT-29/Fibro cocultures produced an average of 45 tissue masses per 5-ml sample, 32 that were 1 to 3 mm in diameter, and 13 that were more than 3 mm in diameter (Figure 11B). In contrast, HT-29KM/Fibro cocultures formed tissue masses that were all greater than 3 mm in diameter (Figure 11B). Since the internal diameter of the collection port was 5 mm, the size of larger tissue masses was estimated with a centimeter ruler prior to removal from the vessels.

DISCUSSION

The ability to construct a large-scale, three dimensional, *in vitro* differentiation model affords a vast array of possibilities for cell biological investigations. The differentiation evidenced in HT-29 and especially HT-29KM cocultures suggests that many aspects of differentiation may be displayed, under proper conditions, in otherwise undifferentiated adenocarcinoma cell lines. The presence of 1) cellular organization and ordering of tissue, 2) development of glandular structures, 3) appearance of cellular morphologies not previously observed in the initial inoculum (signet, goblet, and columnar), 4) production of secretory product (mucin) and apical microvilli and polypoid formations collectively signify a multiphase differentiation process that is the product of the cellular components and physical orientations afforded by the culture conditions. Furthermore, differentiation is achieved without the addition of traditional inducing agents but rather is controlled by the nature of the cell populations.

The importance of a successful large-scale, three-dimensional *in vitro* culture system for intestinal epithelial cells cannot be overemphasized. Colorectal carcinoma is the second most prevalent and lethal cancer in the U.S., with an estimated 60,000 mortalities in 1987.(34) No new effective chemotherapeutic agents specific for colorectal carcinoma have been developed in the past decade. It is clear that some colon carcinomas may differentiate, terminally divide, and die when treated with certain toxic differentiating compounds.(35) These compounds are ineffective in clinical treatment, however, either because they are too toxic or because they break down before reaching their target. If it were known which substances control the maturation and proliferation of colon epithelial cells, then larger doses of those factors might inhibit the further growth and development of colorectal carcinomas.(31) In order to facilitate differentiation in a manner representative of the *in vivo* environment, without changing the base sugar of the growth medium(25,37) or using chemical induction,(10,24) HT-29 and HT-29KM were cocultivated with mixed normal human colon fibroblasts in RWVs. Our

HT-29 cell line did not differentiate when it was implanted in nude mice. HT-29 cultured with normal human colon fibroblasts in RWVs exhibited organization and polarity because it grew over fibroblasts that, for the most part, remained closely attached to the microcarrier bead surface. As the polypoid masses grew away from the fibroblast surface, they broke off and formed spheroids. Furthermore, our HT-29 cell line did not produce signet ring cells in either the RWV or in the nude mouse, and thus our HT-29 line may be different from that used by Richman and Bodmer.(30) HT-29KM, however, demonstrated epithelial polarity and cellular organization, and produced glands and signet ring cells when cultured in the RWV with mesenchymal cells and *in vivo* in the nude mouse. Thus, we conclude that the RWVs support the development of tissues that are remarkably similar to structures that are formed *in vivo*.

The most notable of the results indicates that neoplastic colonic epithelium grows in an enhanced fashion in the presence of mixed human colonic fibroblasts in this culture system. The colonic adenocarcinomas tested grew as tumorous colonic spheroids with minimal differentiation and negligible necrosis in the absence of colonic fibroblasts. This finding may present a new model system in which to test potential antitumor compounds and immunotherapeutic agents.

Our results may have broad implications for other disciplines. First, if large, differentiated masses of epithelial tissues grow in RWVs, then it ultimately may be possible to use these vessels to propagate even more complex tissues, possibly organs. The RWV strategy offers obvious advantages since the use of autologous tissue minimizes chances of rejection. Second, the availability of large masses of differentiated tissues may provide the opportunity to harvest growth and differentiation factors in higher yield than achieved in standard bioreactors and thus facilitate their isolation and study. Such factors are not isolated easily from tissues *in vivo* because the presence of diverse types of cells confounds isolation attempts. Through improved control of the mesenchymal elements, it is conceivable to isolate growth factors at

higher concentration. Further, if induction signals are not secreted but rather transmitted from cell-to-cell,(9) the availability of larger tissues in culture will facilitate identification of intermediary gene products and peptides under controlled conditions. Thus, this new model system has important implications for studies in organogenesis as well as oncology.

Table 1

Maximum Cell Numbers In Different Culture Conditions^a

<u>Condition</u>	<u>HT-29</u>	<u>HT-29KM</u>	<u>Normal Colon Fibroblasts</u>
1. Monoculture in nonadherent dishes	NG ^b	NG ^b	NG ^b
2. Monoculture in nonadherent dishes on microcarriers	$\sim 1.0 \times 10^6$	$\sim 1.0 \times 10^6$	$\sim 0.3 \times 10^6$
3. Monocultures in RWVs on micro- carriers	2.7×10^6	3.3×10^6	0.5×10^6
4. Coculture in RWVs on micro- carriers with normal colon fibro- blasts	9.3×10^6	10.9×10^6	---

^anumber of cells/ml

^bNG = no growth

FIGURE LEGENDS

Fig. 1. Karyotypic analysis of HT-29 (A) and HT-29KM (B).

Fig. 2. A, Growth curves of normal colon fibroblasts, HT-29, and HT-29KM grown on microcarriers in RWV. B, HT-29 cocultivated with normal colon fibroblasts on microcarriers in RWV. C, HT-29KM cocultivated with normal colon fibroblasts on microcarriers in RWV.

Fig. 3. SEM and histological comparison of normal colon fibroblasts, HT-29, and HT-29KM in RWVs. SEMs of normal colon fibroblasts, 300x (A), HT-29, 310x (C), and HT-29KM, 320x (E). A, C, E, Tissue masses (arrows) grown on microcarriers (triangles) in RWVs. 10 μ histological cross sections of microcarrier bead packs stained with H&E (B, D) or mucicarmine (F). Each figure shows a cell mass of normal colon fibroblasts (B), HT-29 (D), or HT-29KM (F) growing on or between microcarriers, 200x.

Fig. 4. Histological comparison by H&E of monocultured normal colon fibroblasts, HT-29, and HT-29KM vs. coculture in RWVs. 10 μ H&E-stained cross sections of microcarrier bead packs (triangles) showing normal colon fibroblasts (A), HT-29 (B), and HT-29KM (D) in monoculture, 200x. 10 μ H&E-stained cross sections of HT-29 (C) and HT-29KM (E) in coculture, 200x. In contrast to the growth of HT-29 in monoculture (B), when cocultivated with normal colon fibroblasts, HT-29 forms polypoid structures (C) which are similar to *in vivo* polyps. Arrows at the base of the polyp indicate fibroblasts. In contrast to the monocultures of HT-29KM (D), HT-29KM in coculture grows over beads and differentiates forming signet ring cells (small triangles), glandular structures, and cellular organization (E).

Fig. 5. Histological comparison by mucicarmine of monocultured normal colon fibroblasts, HT-29 and HT-29KM vs. coculture in RWVs. 10 μ mucicarmine-stained cross sections of

microcarrier bead packs (*triangles*) showing normal colon fibroblasts (A), HT-29 (B), and HT-29KM (D) monoculture, 200x. 10 μ mucicarmine-stained cross sections of HT-29 (C) and HT-29KM (E) cocultures, 200x. In contrast to the growth of HT-29 in monoculture (B), cocultivation of HT-29 with normal colon fibroblasts results in polypoid structural development (C) which is similar to that seen *in vivo*. Note fibroblasts (*arrows*) on the surface of microcarriers (*triangles*). In contrast to the undifferentiated growth of HT-29KM (D), many packets of mucin (*arrows*) (differentiated secretory product), cellular differentiation, and organization can be seen in HT-29KM cocultures (E).

Fig. 6. Comparison TEMs of HT-29 and HT-29KM in RWV monoculture, 10,300x (A, B) and 17,000x (C). Note the multiple nuclei and nucleoli (A, B), poor cellular junctions (A, C), and overall aberrant cell morphology. HT-29KM grown in monoculture on microcarriers in RWVs, 8,100x (D, E) and 10,300x (F). Note the irregularity of the nuclei and nucleoli and large open spaces at the cell-cell junctions (D, E). Characteristic intraglandular structure occasionally found in HT-29KM F.

Fig. 7. Cocultures of HT-29 in RWVs analyzed by light microscopy, SEMs, and histological staining. A, B, Light microscopy of HT-29 cocultured with normal colon fibroblasts (note polyps, *arrows*), 200x. SEMs of HT-29 cocultured on microcarriers, 300x (C) and 1,000x (D). C, Normal colon fibroblasts are seen growing on microcarriers with a large tumor polypoid atop the bead pack. D, SEM 1,000x of C showing the cell-cell interaction between fibroblasts (*arrows*) and tumor cells. 10 μ mucicarmine-stained cross sections of HT-29 cocultivated on microcarriers (*triangles*), 200x (E) and 400x (F). E, Polypoid (*arrows*) with center invasion of normal colon fibroblasts similar to *in vivo* tumor polyps.

Fig. 8. Cocultures of HT-29KM in RWVs analyzed by light microscopy, SEMs, and histological staining. Light microscopy of HT-29KM cocultivated with normal colon fibroblasts, 200x (A)

and 100x (B). A. Microcarrier (*triangle*) covered with fibroblasts; pseudo gland (*arrows*) which has formed from cells. B, A large differentiating mass of HT-29KM tumor cells; large pseudo gland *arrows*. C, D, SEMs of HT-29KM cocultivated on microcarriers with normal colon fibroblasts. D, Closeup of a microcarrier with elongated fibroblasts and an over layer of HT-29KM tumor cells. E, F, 10 μ H&E- and mucicarmine-stained cross section of HT-29KM coculture 200x. Note the signet ring cell formation in the upper right (*small triangle*). F, Two microcarriers (*large triangle*) are visible and fibroblasts (*arrows*) can be seen growing across the surface. Microcarrier (*triangle*) from which sinusoids radiate (*arrows*).

Fig. 9. Histological comparison of mouse xenografts of HT-29 and HT-29KM vs. HT-29 and HT-29KM cocultivated in RWVs. 5 μ H&E-stained cross sections of HT-29 (A) and HT-29KM (C) mouse xenografts, 100x. HT-29 shows no differentiation and HT-29KM shows areas of limited cellular differentiation and signet ring cell development (*lower left*). 10 μ H&E-stained cocultures of HT-29 (B) and HT-29KM (D), 200x. B, A large polypoid growing out from the microcarrier (*far left, triangle*). Arrows mark the apex of the polypoid. D, Signet ring cell formations (*arrows*). In contrast to C, the tissue in D shows structural organization and differentiation. Multiple signet ring cells and glandular formations are visible. In addition, a line of columnar epithelial cells can be seen to the immediate left of the microcarrier in the center of the photograph (D).

Fig. 10. TEMs of HT-29KM in RWV coculture. HT-29KM cocultivated with normal colon fibroblasts, 5,400x (A) and 9,000x (B, C). Note the regularity of the nuclei and reduction in multiple nucleoli as compared to Fig. 6 D and E. Note the presence of an apical microvillus border (A, B, C) and intercellular gland structures (F). HT-29KM cocultured with normal colon fibroblasts, 15,000x (D), 10,300x (E), and 7,000x (F). Note the columnar epithelium, tight cellular junctions, and sinusoid formation in D. Normal colon fibroblasts layered over a

microcarrier and large amounts of extracellular matrix produced in coculture (E). F, Inter-glandular structure formed by the junction of several cell borders with internal microvilli.

Fig. 11. Five-ml samples were harvested from cocultures in the RWV at 15-19 days post initiation. Samples were plated in 35-mm petri dishes that contained a 2-mm grid. The number of polypoid and gland structures were quantitated against the grid in the dish (A). Samples described in (A) were measured on a 2-mm gridded petri dish to determine the relative size of the tissue masses (B). Tissue masses larger than 0.5 cm were not measured in petri dishes but with a centimeter ruler due to the size of the extraction port.

A



B

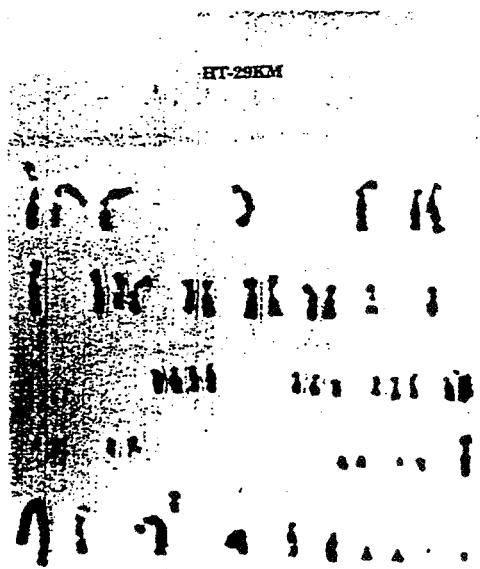


Figure 1

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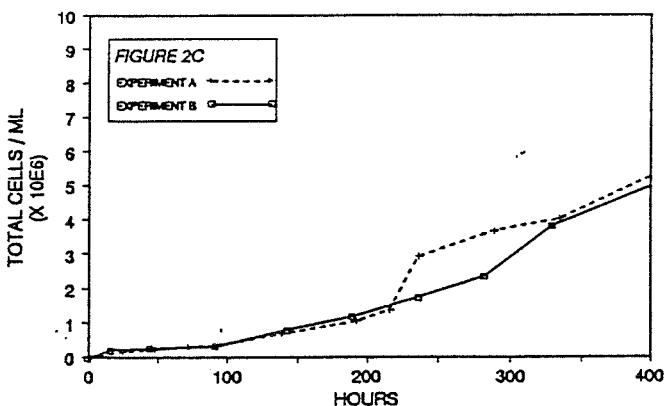
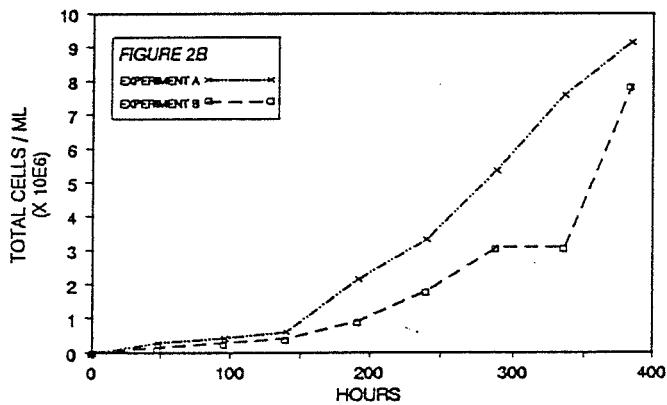
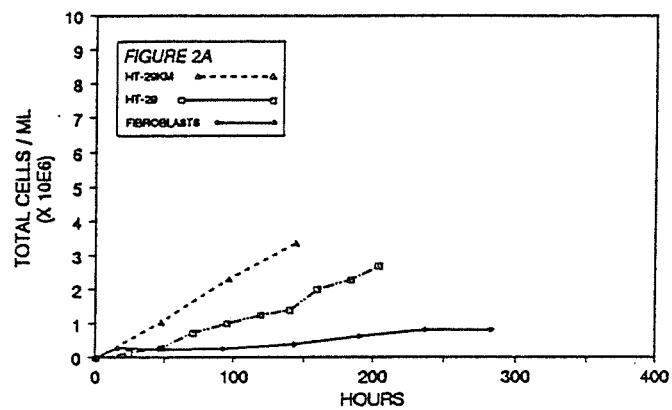


Figure 2

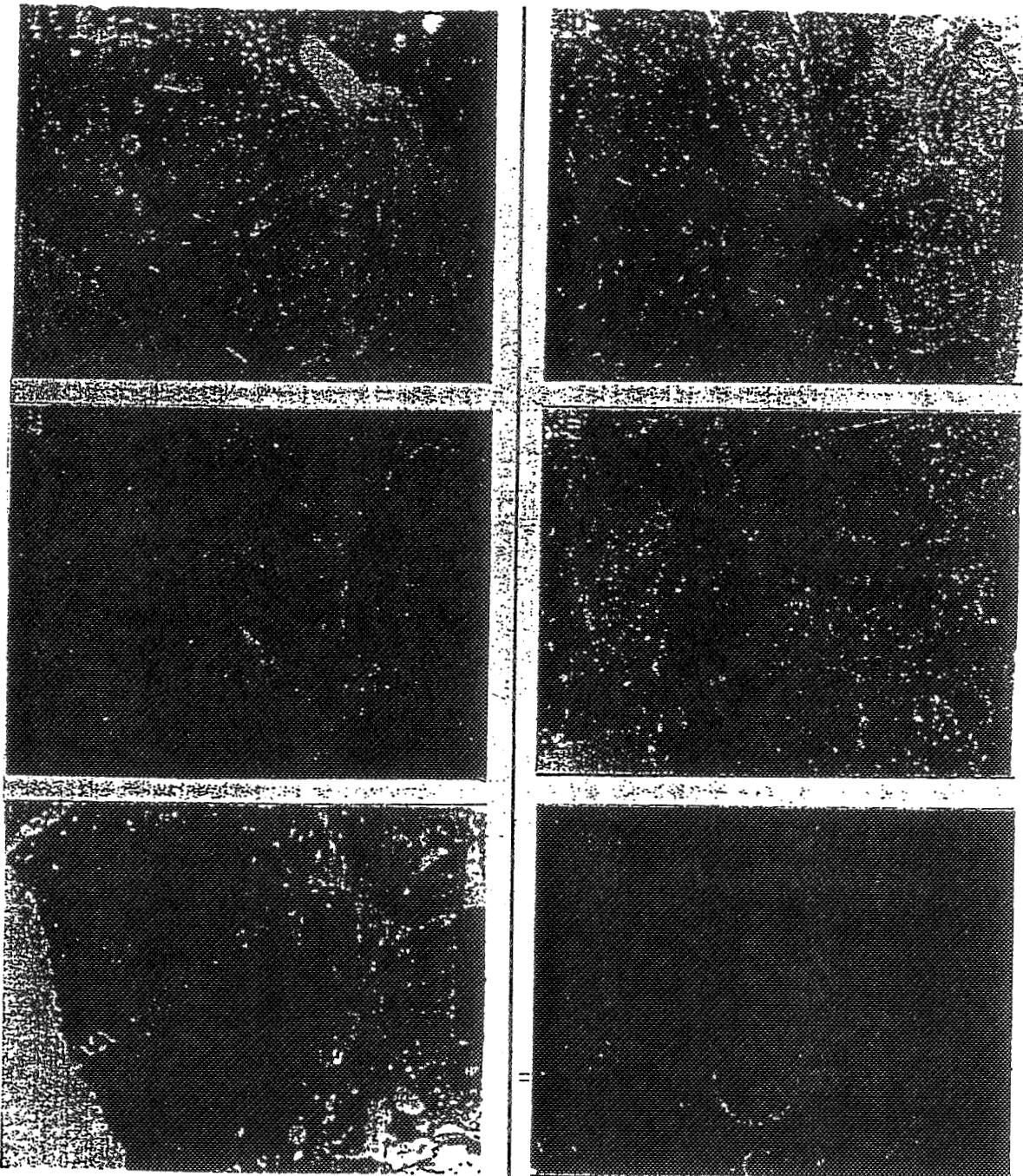
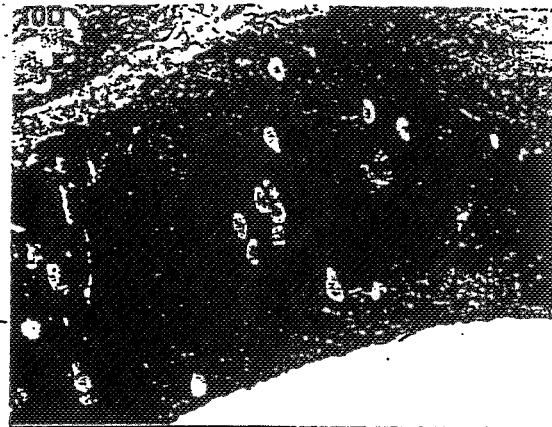
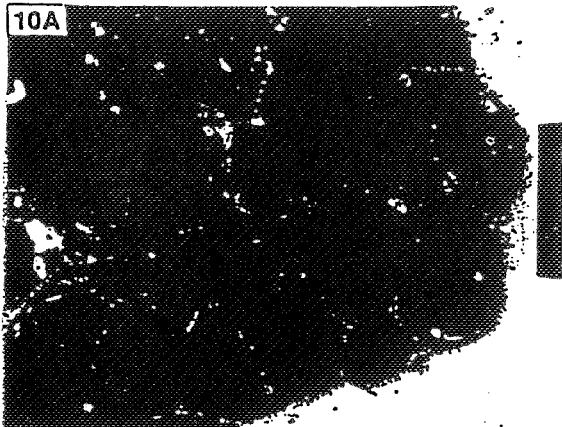
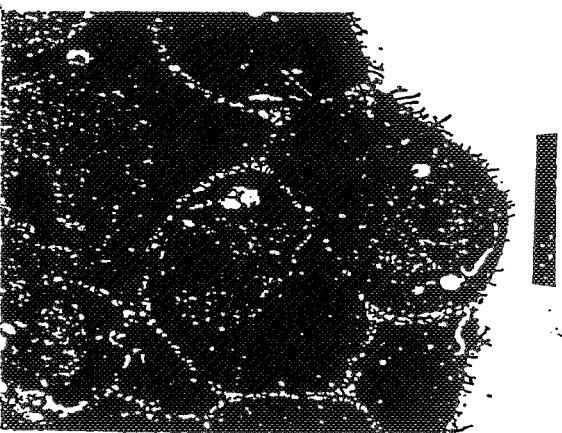


Figure 6

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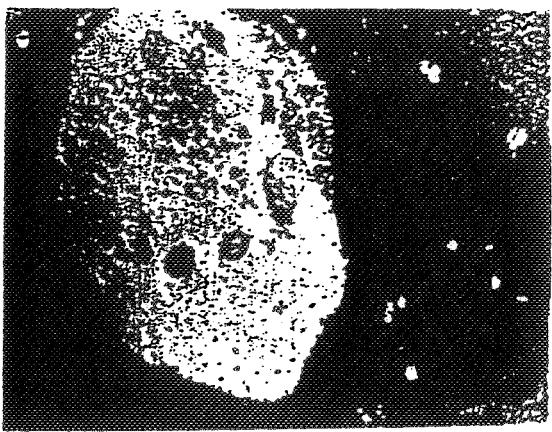
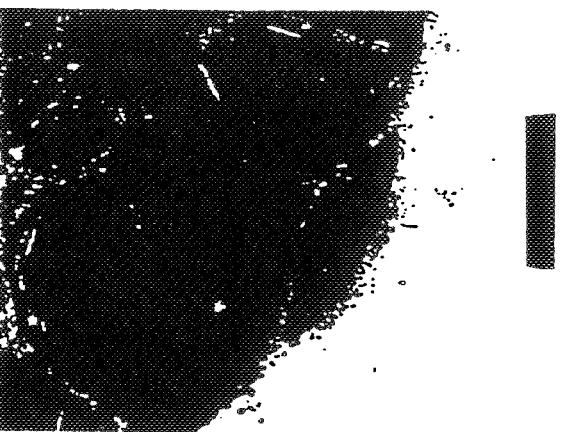


Figure 10

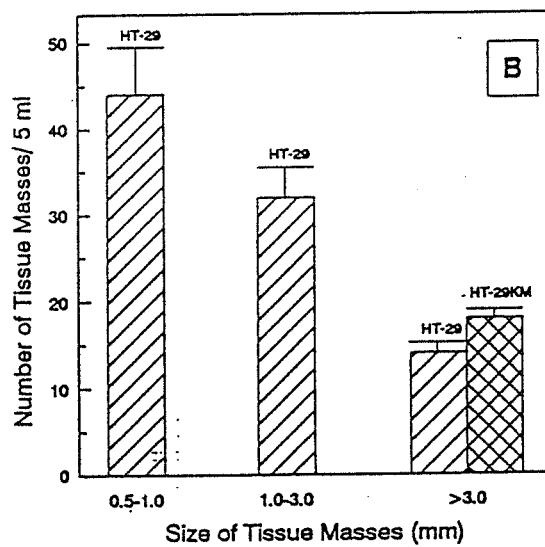
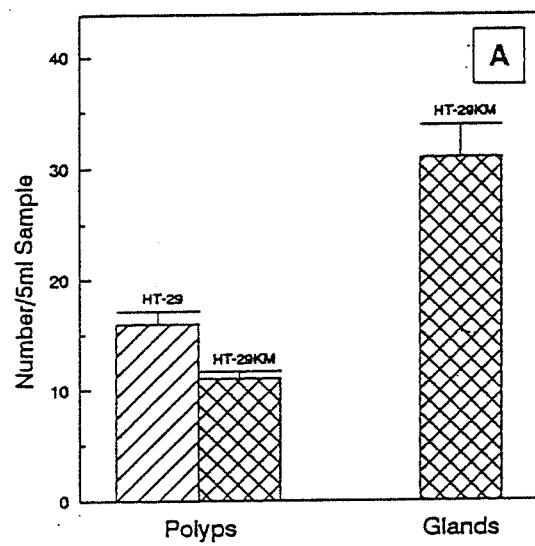


Figure 11

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SIMULATED MICROGRAVITY DOES NOT ALTER EPITHELIAL CELL ADHESION TO MATRIX AND OTHER MOLECULES

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ABSTRACT

Microgravity has advantages for the cultivation of tissues with high fidelity; however, tissue formation requires cellular recognition and adhesion. We tested the hypothesis that simulated microgravity does not affect cell adhesion. Human colorectal carcinoma cells were cultured in the NASA Rotating Wall Vessel (RWV) under low shear stress with randomization of the gravity vector that simulates microgravity. After 6 - 7 days, cells were assayed for binding to various substrates and compared to cells grown in standard tissue culture flasks and static suspension cultures. The RWV cultures bound as well to basement membrane proteins and to CEA, an intercellular adhesion molecule, as control cultures did. Thus, microgravity does not alter epithelial cell adhesion and may be useful for tissue engineering.

INTRODUCTION

Production of complex tissues requires that individual cells recognize and adhere to other cells and to extracellular matrix and basement membrane molecules. Microgravity may be useful in engineering new tissues *in vitro* because the lack of sedimentation may permit the formation of tissues with high fidelity to their *in vivo* counterparts. However, microgravity must not alter the ability of cells to recognize and adhere to extracellular matrices and intercellular ligands if it is to be used to construct tissues. The purpose of this study was to test the hypothesis that microgravity does not affect cell adhesion. This was tested in the NASA Rotating Wall Vessel (RWV) in which cells suspended in aqueous medium rotate around the horizontal axis under conditions of low shear stress. The rotation randomizes the gravity vector and simulates some aspects of microgravity. Human colorectal carcinoma cells were used as a model for epithelial cell adhesion because they use the same adhesion receptors as normal epithelium. Cells were cultured for short (6 - 7 days) or long (23 days) periods and assayed for binding to standard adhesion molecules. Although the amount of CD44 was decreased in long term cultures, cells grown in simulated microgravity had the same binding characteristics as did cells grown in standard tissue culture flasks. Thus, microgravity may be useful for tissue engineering.

METHODOLOGY

Cell Lines

The HT-29KM cell line is a sub-line of the HT-29 cell line /1/. The cell line KM-12c was established at the U. T. M.D. Anderson Cancer Center /2,3/. MIP-101 was established by Niles et al. /4/, while CCL 188 was obtained from the American Type Culture Collection (Rockville, MD). All cells were grown and maintained at 37°C in a humidified atmosphere containing 5% CO₂. HT-29KM, CCL 188, and KM-12c were maintained in Dulbecco's minimal essential media (DMEM) supplemented with 10% fetal calf serum, and 0.30 mg/ml of L-glutamine. MIP-101 cells were grown in RPMI-1640 media supplemented with 7.5% fetal calf serum, and 0.30 mg/ml of L-glutamine. All cell lines were cultured in the presence of 100 units/ml of penicillin and 100 µg/ml of streptomycin. Medium was changed every third day and monolayers of cells (85-90% confluent) were harvested by mild trypsinization. After harvesting, cells were counted using a hemocytometer and viability determined by trypan blue dye exclusion. Cells were routinely 85-90% viable. All culture media and sera were obtained from

Sigma Chemical Co., St. Louis MO. Cultures were routinely tested for the presence of mycoplasma by staining with Hoechst dye 33258 and were negative.

Monolayer Cultures

Cells were seeded into T25 flasks (Costar) or nonadherent bacterial Petri dishes (Falcon) at 3×10^5 cells/ml in their standard culture medium. Cells were cultured for the indicated number of days, harvested by trypsinization, and then tested in the adhesion assay.

Rotating-Wall Vessel (RWV) cultures

The RWV is a 125 ml vessel that rotates around the horizontal axis. Cells were added to dextran microcarriers coated with collagen (Cytodex-3 beads) (Pharmacia, Piscataway, NJ) at 2×10^5 cells/ml (or a total of 5.0×10^7 cells), which resulted in a starting concentration of 10 cells per bead since the concentration of beads was 5 mg/ml. After inoculating the RWV, the cultures were allowed to grow for 48 hours before the medium was changed. Thereafter, the medium was changed every 20 to 24 hours; as the metabolic requirements of the cultures increased, fresh medium was supplemented with an additional 10 to 200 mg/dl of glucose. Cells were harvested for the adhesion assays by dissociation with collagenase and DNase.

Adhesion Assay

Quantitation of the binding between tumor cells and the various substrates was determined by a modification of the method described by Hostetter et al. /5/. The substrates were dried, at a concentration of 1 μg per well in phosphate buffered saline (PBS), onto 96 well plates (Costar, Cambridge, MA) for 24 hours at 37°C and the plates washed with PBS. The free binding sites on the plastic were blocked with 4% BSA in PBS for 2 hours at 37°C and the plates washed with PBS. The cells were prelabelled with 300 μCi ^{51}Cr (Na_2CrO_4 , specific activity 450 mCi/mg, New England Nuclear, Boston, MA) for 1 hr at 37°C . The cells were washed with calcium-free PBS, harvested by mild trypsinization and plated at concentrations of 1×10^4 cells per well in the coated plates. After centrifugation (30g x 5 mins), the plates were incubated for two hours at 37°C . Nonadherent cells were removed by three washes with 0.5% BSA in PBS and the remaining cells were solubilized with 1M NaOH and radioactivity collected on cotton-tipped swabs. These were counted for ^{51}Cr using a Tracor Analytical gamma counter. The percent of cells bound was defined as the ratio of the mean number of counts in an experimental well divided by the mean number of counts plated in each well. The concentration of 1 $\mu\text{g}/\text{well}$ of CEA and other substrates was chosen from titration studies with KM-12c as well as repetitive experiments which indicated that optimal and reproducible binding was achieved at that concentration.

RESULTS

Growth in the RWV Compared to Standard Tissue Culture Systems

Human colorectal carcinoma cells in the RWV routinely achieved cell concentrations of $0.5 - 1.5 \times 10^6$ cells/ml or greater in short term cultures of up to 10 days (Figure 1A). When cells were cultured for longer periods (20 - 30 days), they grew to greater concentrations before plateauing (HT-29KM to $4 - 5 \times 10^6$ cells/ml /1/ or MIP-101 cells to 5×10^6 cells/ml at 23 days). These cell densities are greater than the $0.9 - 2.0 \times 10^6$ cells/ml achieved with these cell lines in standard monolayer cultures in tissue culture flasks.

Growth of Human Colorectal Carcinomas in the RWV

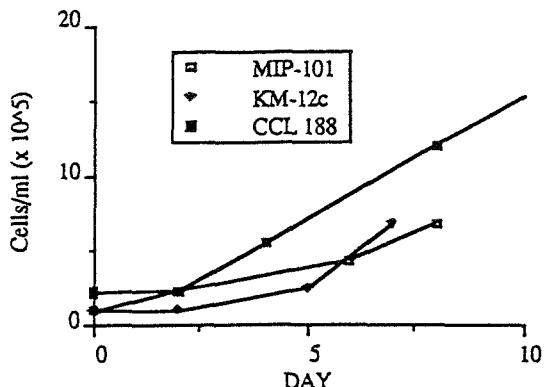


Fig. 1 Cells were added to Cytodex 3 microcarrier beads and cultured in the RWV as described in the "Methodology."

Adhesion to Laminin and Collagen in Standard Culture Systems

Cells cultured in standard monolayer cultures were assessed for their ability to bind to proteins in extracellular matrix and basement membranes as well as to CEA, an intercellular adhesion molecule. CCL 188 and KM-12c cells bind to laminin and CEA while HT-29KM and MIP-101 cells bind to laminin alone (Table 1). None of the colorectal carcinoma cell lines bind to fibronectin.

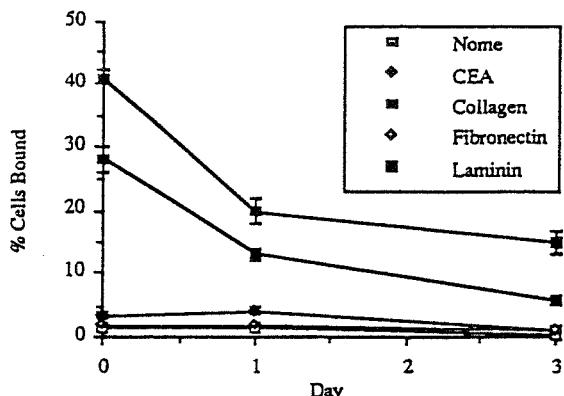
TABLE 1 Adhesion of Human Colorectal Carcinoma Cells Cultured in Standard Tissue Culture Flasks To Molecules Attached To a Solid Phase.

Cell Line	CEA Production	% Cells Bound (Mean \pm SEM) ^c			
		None	BSA	Fibronectin	Laminin
HT-29KM	300	1 \pm .1	1 \pm .2	2 \pm .1	34 \pm 1
CCL 188	97	1 \pm .1	2 \pm .3	1 \pm .1	43 \pm 4
KM-12c	1950	3 \pm 2	3 \pm 1	12 \pm 7	60 \pm 7
MIP-101	0	10 \pm 1	9 \pm 1	11 \pm 1	80 \pm 6

Bold print represents a significant difference from the negative controls of $P < 0.001$ by Scheffe test.
CEA Production is in ng/ml of medium.

When the effect of time in culture was examined, there was a relative decrease in the adhesion of cells to collagen or laminin up to 3 days after plating in monolayer cultures. Both KM-12c (Figure 2) and HT-29KM cells (Figure 3) had a relative decrease in the number of cells that bind to collagen and laminin but both cell lines had significantly greater binding to collagen and laminin than they did to CEA, fibronectin, or the control protein BSA (Figures 2 and 3). Similarly, when cells were plated on bacterial Petri dishes so that they remain in suspension but do not attach to a surface, KM-12c cells maintained their ability to bind to collagen and laminin (Figure 2); however, HT-29KM lost its ability to bind to collagen and laminin within three days (Figure 3). Both cell lines sustained a large loss of viability when cultured in static suspensions for more than three days that precluded any further evaluation. Interestingly, the adhesion to CEA by KM-12c cells was not great in either the monolayer or static suspension culture system (Figure 2). This may be because the expression of CEA by recently plated cells is low and only increases as cells attain the plateau phase of growth. Nonetheless, cell adhesion in RWV cultures were compared to adhesion in standard monolayer cultures because the static suspension cultures were largely nonviable.

Fig. 2A.
KM-12c Cells In Standard Monolayer Culture



2B.
KM-12c Cells in Static Suspension Culture

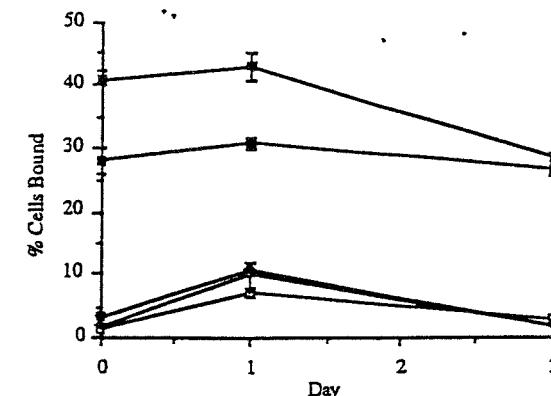
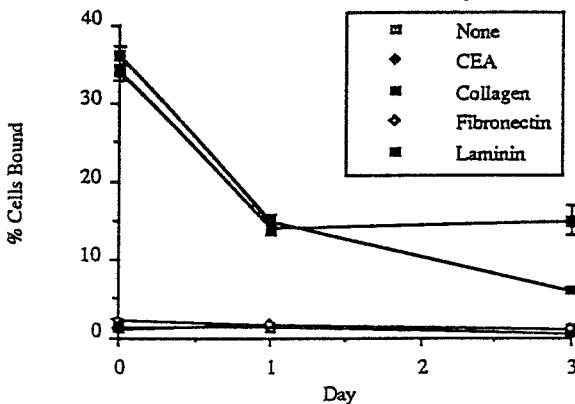


Fig. 2

Fig. 3A.
HT-29KM Cells In Standard Monolayer Culture



3B.
HT-29KM Cells in Static Suspension Culture

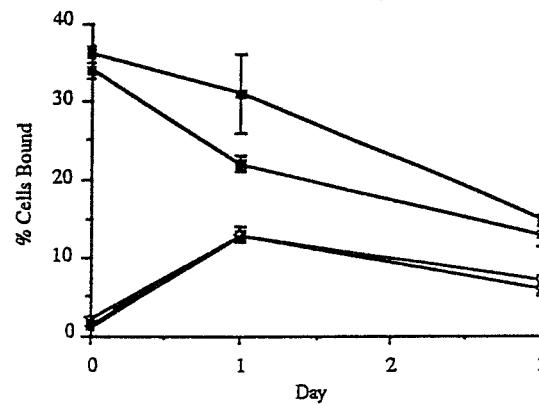


Fig. 3

Figs 2 and 3. Cells were plated in standard T25 tissue culture flasks (monolayer cultures) or in bacterial Petri dishes (static suspension cultures) as described in "Methodology". Cells were harvested and then assayed for binding to various adhesion substrates. Cells were not viable in the static suspension cultures after the third day so that experiments could not be performed with those cultures. However, cells retain their ability to adhere to basement membrane molecules even when cultured in static suspension cultures.

Adhesion in the RWV Cultures

When cultures of CCL 188, KM-12c, and MIP-101 cells were compared to paired aliquots of cells grown for six or seven days in standard monolayer cultures, there were no significant differences in the ability of cells grown in the RWV to bind to collagen or laminin (Table 2). CCL 188 and KM-12c cells maintain their ability to bind to CEA when cultured in the RWV for up to six days (Table 2). On longer culture in the RWV, MIP-101 cells maintain their ability to bind to collagen and laminin (Table 3). However, the nonspecific binding to BSA also increases. In addition, the expression of CD44, one of the adhesion receptor molecules on MIP-101 (manuscript submitted), decreases from a mean fluorescence intensity of 439 for monolayer culture cells to 289 for cells cultured for 23 days in the RWV.

TABLE 2 Culture In The RWV Does Not Inhibit The Ability Of Epithelial Cells To Bind To Inter- and Extracellular Ligands

Substrate To Which Cells Adhere:	% CELLS BOUND (MEAN ± SEM)					
	KM-12c		MIP-101		CCL 188	
	RWV	Control	RWV	Control	RWV	Control
None	6 ± 1	3 ± 1	14 ± 1	10 ± 1	20 ± 2	1 ± .1
CEA	32 ± 3	6 ± .3	13 ± 1	9 ± 1	31 ± 3	16 ± 1
Collagen IV	58 ± 2	26 ± 1	29 ± 1	61 ± 2	47 ± 4	54 ± .6
Fibronectin	3 ± 1	4 ± 1	11 ± 1	11 ± .3	22 ± 2	1 ± .1
Laminin	57 ± 9	19 ± .4	53 ± 1	63 ± 2	25 ± 2	43 ± 4

Cells were started from the same stock and harvested from standard monolayer or RWV cultures after 6 - 7 days and analyzed for their ability to adhere to various ligands. Bold Print represents significant differences between the control (None) group and experimental groups of at least P<0.01 by Scheffe test. While the magnitude of the adhesion to the various substrates may change with different culture conditions, the ability to bind is not impaired in the RWV culture. This suggests that cells will be able to associate in actual microgravity as well as they do in unit gravity.

TABLE 3. The Effect of Long Term (23 Day) RWV Culture on MIP-101 Cell Adhesion

Substrate	% Cells Bound (Mean ± SEM)
None	11 ± 2
BSA	18 ± 3
Collagen	23 ± 1
Laminin	34 ± 1

MIP-101 cells were cultured in the RWV for 23 days when they had attained a concentration of 5.23×10^6 cells/ml and then assayed for binding to collagen and laminin. Assay conditions as described in Table 2.

DISCUSSION

The present results indicate that short term cultures of six to seven days in simulated microgravity do not alter the ability of epithelial cells to bind to adhesion molecules. This is important because it suggests that cells will be able to associate in actual microgravity to create structures that may be either too fragile for culture in unit gravity or that require three-dimensional architecture for function. This observation is extremely relevant to attempts to cultivate normal tissues that have so far been difficult to grow in unit gravity systems. An example where this may be important is the cultivation of normal colonic mucosa. Cultures of normal colonic cells do not persist for more than 5 days usually when the epithelial cells are separated from their surrounding stroma. However, in the host these epithelial cells live in a complex three-dimensional structure, the crypt of Lieberkuhn. This crypt is a layer of cells, one cell thick, that forms an outpouching or balloon-like structure into the surrounding stroma. Crypts can be isolated intact and placed in culture but they collapse under their own weight in aqueous medium in unit gravity. Maintenance of the three-dimensional structure does not promote crypt growth because crypts embedded in collagen or Matrigel matrices keep their structure but die as quickly as isolated cells. The viable crypt structure probably requires the intimate interaction of the stromal cells with the epithelial cells in an environment that maintains crypt architecture. Microgravity may permit the interaction of stromal and epithelial cells to sustain crypt cell proliferation and promote the formation of new crypts. This form of tissue engineering can only occur if cells maintain their ability to recognize and associate with the proper adhesion molecules.

- It is not clear what the importance of the increase in nonspecific binding is on long-term cultures. Adhesion to collagen and laminin in colonic cells is primarily mediated by integrins /6/. These were not measured in the present experiments. However, CD44 is another adhesion receptor that is also involved in adhesion to collagen /7/ and migration on basement membranes /8/. We have shown that monoclonal antibodies to CD44 block the attachment of CCL 188, KM-12c, and MIP-101 cells to collagen and laminin (data not published). Therefore, the decrease in the relative expression of CD44 by MIP-101 cells may be an important observation. Clearly, this alteration does not decrease the ability of these cells to bind to laminin, although attachment to collagen was slightly decreased. Compensatory mechanisms, e.g., the adhesion mediated by integrins, may be activated to maintain cellular adhesion.

In summary, the present results suggest that epithelial cells will be able to recognize and associate properly in a microgravity environment. This presents exciting opportunities for tissue engineering when access to microgravity is achieved.

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**Simulated Microgravity Improves
Production of Colonic Growth Factors**

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**AIAA Space Programs
and Technologies Conference
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SIMULATED MICROGRAVITY IMPROVES PRODUCTION OF COLONIC GROWTH FACTORS

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ABSTRACT

The purpose of this study was to determine whether simulated microgravity may be used to create masses of cells that are similar in function to tissues that exist in animals and humans. Human colon carcinoma cells were cultured with normal human colonic fibroblasts in a Rotating Wall Vessel (RWV) designed by the NASA/JSC Biotechnology Program. The RWV rotates cells so that they are suspended in a one-fifth gravity field under low shear stress. Cells cultured in the RWV not only look like cancer growing in nude mice but also produce factors that stimulate selective growth of normal mouse colonocytes. Tumor cells grown in conventional monolayers or in roller bottles even in the presence of normal human colonic fibroblasts do not either form masses of cells that look like tumors grown in mice or produce colonic growth factors. These data suggest that actual microgravity will permit construction of novel tissues in the future.

INTRODUCTION AND PURPOSE

The microgravity of Space Station may permit cultivation of mammalian cells in a three dimensional

aqueous matrix that creates tissue in vitro functionally and structurally similar to tissue formed in vivo. The purpose of this study was to determine whether growth factor production by human colonic cells is enhanced in simulated microgravity compared to conventional tissue cultures or to tissues from patients. If simulated microgravity conditions induce growth factor production similar to that observed in vivo and not produced in other culture systems, then actual microgravity may be even more advantageous for the production of tissues in culture. Microgravity was simulated for batch tissue culture in the Rotating Wall Vessel (RWV) which is a zero head space fluid-filled clinostat designed by NASA. The RWV simulates certain aspects of microgravity because it suspends particles under lower shear stress than conventional batch culture systems do. Human colonic carcinoma cell lines co-cultured with human fibroblasts form tissue masses in the RWV that are morphologically similar to the carcinoma grown in vivo in athymic nude mice¹. We have also previously shown that freshly isolated human colorectal adenocarcinomas implanted in the wall of the large bowel of athymic nude mice release soluble factors that selectively stimulate proliferation of goblet cells in the normal murine colonic mucosa overlying the tumor implants². Goblet cells produce mucin

that lubricates and binds fecal contents for excretion and are easily distinguished from the other epithelial cells that line the crypts of Lieberkühn that line the mucosa of the large bowel. In the present study medium was conditioned by HT-29 human colon carcinoma cells growing on microcarrier beads in either the simulated microgravity of the RWV or in the higher shear stress field of roller bottles that are a conventional culture vessel. The conditioned media were then tested for their effects upon the production of goblet cells in colonic mucosa. The results suggest that RWV cultures of HT-29 and a subline, HT-29KM, like freshly isolated human colorectal carcinomas, produce a factor that stimulates the production of goblet cells and that this factor is not detectable in media from roller bottle cultures.

METHODS

0.15M phosphate buffered saline (saline), tissue culture medium (alpha MEM with 10% fetal calf serum) or culture medium conditioned by the HT-29 colon carcinoma cell line, its subline HT-29KM, or normal human fibroblasts cultured in the RWV or roller bottles was encapsulated into liposomes and 0.05 ml of 6 μ M of liposomes in PBS was injected into the cecal submucosa of BALB/c mice. The liposomes were used to provide a sustained release of potential growth factors into the colonic submucosa. The culture medium and supernatants were concentrated ten-fold and one ml incorporated into 60 μ M of CGP 19,835 A lipids manufactured by Ciba-Geigy. Five days later the mice were killed and the colons harvested, irrigated with saline, and each cecum fixed in formalin. Paraffin sections were then stained with hematoxylin and eosin and the morphometric analysis was performed in a coded fashion. Forty crypts that were sagitally sectioned over the liposomes were counted per

treatment group. In Exps. 1 and 3 the medium was harvested from cultures in the RWV while in Exp. 2 the conditioned medium was collected from a roller bottle culture of HT-29. Particles in the roller bottle culture were less than 0.5 cm while the RWV cultures contained many particles between 0.5 and 1.0 cm. These particles are aggregates of cells and microcarrier beads. In some experiments India ink, epidermal growth factor (EGF), basic fibroblast growth factor (FGF) or gastrin were incorporated in liposomes as controls.

RESULTS

Fresh medium did not stimulate any changes in crypt cell height or number compared to saline controls. In contrast, India ink caused acute inflammation and increased crypt height, cell number, and goblet cells per crypt without increasing the percentage of goblet cells (Table 1). Media from cultures of HT-29 or its subline HT-29KM in either roller bottles or the RWV increased the height of crypts, the number of cells per crypt, and the absolute number of goblet cells per crypt compared to saline controls (Table 1). However, media from RWV cultures (Exp. 1 & 3, Table 1) selectively increased the percentage of goblet cells whereas media from roller bottle cultures (Exp. 2, Table 1) only increased the total number of crypt cells. These effects of conditioned media were due to factors released by carcinoma cells because media from human fibroblast cell cultures in the RWV did not produce any effect on crypt height or cell number (Table 1).

The factor(s) responsible for the selective expansion of goblet cells have not yet been isolated. However, purified growth factors (EGF, FGF, and gastrin) increased crypt height and/or cell number but did not increase the number of goblet cells (Exp. 4, Table 1).

DISCUSSION

Cultivation of established human colon carcinoma cells in the simulated microgravity of the RWV produced a factor that selectively expanded goblet cells in normal mouse large bowel epithelium. Similar cultures in roller bottles did not produce an activity that achieved the same selective expansion of goblet cells, although there was a nonselective trophic effect on the bowel mucosa. While the goblet cell stimulating activity has not yet been isolated and characterized, it is possible that a novel intestinal peptide is responsible for this activity. It is also possible that previously described factors acting either alone or in concert may have have similar effects on selectively expanding the different subsets of colonocytes. EGF, bFGF, and gastrin are growth factors that are known to stimulate both hypertrophy and hyperplasia in normal colon mucosa in both rodents and humans. However, purified EGF, bFGF, or gastrin did not selectively expand the goblet cell subset at concentrations that were trophic for colonic mucosa. Two of the growth factors (EGF and gastrin) caused significant colonocyte proliferation since there were approximately 50% more cells per crypt in mice treated with these two growth factors than in crypts from control or bFGF-treated mice (Table 1, Experiment 4). Since freshly isolated human colon carcinomas have the same effect on goblet cells as the RWV HT-29 colon cell line cultures did², the simulated microgravity of the RWV may provide a better three-dimensional environment for isolating novel factors than conventional batch culture systems. These data suggest that actual microgravity may provide an excellent opportunity for the isolation of novel growth factors.

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TABLE 1 Liposome Treatment

		Crypt Height ^a	Crypt Cell Number ^b	Crypt Goblet Cell Number ^c	% Goblet Cell ^d
Exp. 1 RWV	Saline	33.6 ± 0.9	36.3 ± 1.1	3.0 ± 0.2	8.7 ± 0.5
	India Ink	76.1 ± 2.1	66.9 ± 2.5	5.3 ± 0.4	8.0 ± 0.6
	Medium	32.2 ± 0.8	35.3 ± 0.9	3.8 ± 0.2	10.9 ± 0.6
	HT-29 Medium	44.8 ± 0.9	42.5 ± 1.3	5.7 ± 0.3	13.6 ± 0.8
Exp. 2 Roller Bottle	Saline	66.2 ± 2.8	32.1 ± 1.5	5.1 ± 0.3	16.4 ± 1.1
	Medium	74.2 ± 2.1	33.1 ± 0.9	3.6 ± 0.2	11.0 ± 0.7
	HT-29 Medium	93.2 ± 2.3	47.4 ± 1.0	7.9 ± 0.3	16.7 ± 0.7
Exp. 3 RWV	Saline	58.5 ± 1.3	28.7 ± 0.7	6.0 ± 0.2	21.2 ± 0.9
	Medium	64.4 ± 1.4	33.4 ± 1.3	6.5 ± 0.3	19.5 ± 0.6
	Fibroblast Medium	56.9 ± 1.0	30.3 ± 0.7	5.6 ± 0.3	18.5 ± 1.1
	HT-29KM/ Fibroblast Medium	90.9 ± 1.7	33.0 ± 1.4	9.8 ± 0.6	25.6 ± 1.4
	Gastrin 1µg	71.2 ± 2.0	34.9 ± 1.1	5.1 ± 0.4	14.5 ± 1.0
Exp. 4	Saline	54.0 ± 2.1	23.3 ± 0.9	5.2 ± 0.4	23.1 ± 1.8
	EGF 1µg	80.1 ± 2.4	36.4 ± 0.8	5.2 ± 0.3	14.8 ± 1.0
	bFGF 1µg	69.2 ± 2.4	29.2 ± 1.1	4.3 ± 0.3	15.3 ± 1.0
	Gastrin 1µg	71.2 ± 2.0	34.9 ± 1.1	5.1 ± 0.4	14.5 ± 1.0

Mean ± SEM. **Bold print-** p< 0.01 compared to saline controls by ANOVA and Scheffe F test. Mice were 6 weeks old in first experiment and nine weeks old in other experiments.

^aMean ± SEM of the height of sagittally sectioned crypts (in 1/250 mm) over submucosal liposomes.

^bCell Number is the Mean ± SEM of the total number of cells in one-half of a sagittally sectioned crypt.

^cGoblet Cell Number is the Mean ± SEM of the number of goblet cells per half crypt.

^dPercentage of Goblet Cells is the goblet cell number divided by the number of cells per half crypt for each individual crypt (Mean ± SEM).



APPENDIX 4: MISCELLANEOUS DATA

Table 1. Glucose Utilization Rates (GUR) For Fibroblasts, HT-29, and HT-29KM in 10^{-13} Mole/Cell/Hour

<u>Cell</u>	<u>STLV</u>	<u>T25 Flask</u>	<u>P<</u>
Fibroblasts	6.7 ± 0.2	5.3 ± 0.5	0.05
HT-29	1.8 ± 0.2	4.0 ± 0.3	0.01
HT-29KM	1.2 ± 0.2	8.0 ± 1.3	0.001

Figure Legends:

Figure 1. Glucose Utilization Rates (GUR) for HT-29 (A) and HT-29KM (B) during monoculture of either carcinoma line, human colon fibroblasts alone, or in coculture of carcinoma cells and fibroblasts. GUR calculated as in Table 1 above. As the cultures increase in cell number the GUR of the coculture decreases to that of tumor cells alone.

Figure 2. Growth of CCL 188 in the RWV and production of TGF β 1. CCL 188 moderately differentiated human colon carcinoma cells were inoculated into a 125 mL RWV (STLV) at 2×10^5 cells/mL in DMEM with 10% FCS and antibiotics. Cell counts determined by enzymatic dissociation at the times indicated. Viability was assessed by trypan blue dye exclusion. TGF β 1 was determined by EIA with monospecific kit from R & D Systems, Inc. CCL 188 cells grew progressively until day 14 then viability cell count decreased with an associated increase in TGF β 1 production. () represents production of TGF β 1 by a similar number of CCL 188 cells grown in monolayer culture in a T25 flask for 4 days.

Figure 3. Growth of KM-12c in the RWV and production of TGF β 1. KM-12c poorly differentiated human colon carcinoma cells were inoculated into a 125 mL RWV at 2×10^5 cells/mL in DMEM with 10% FCS and antibiotics. Cell counts determined by enzymatic dissociation at the times indicated. Viability was assessed by trypan blue dye exclusion. TGF β 1 was determined by EIA with monospecific kit from R & D Systems, Inc. Cell proliferation increased late in this culture associated with a relative decrease in the amount of TGF β 1 produced by KM-12c cells.

Figure 4. Growth of MIP-101 in the RWV and production of TGF β 1. MIP-101 poorly differentiated human colon carcinoma cells were inoculated into a 125 mL RWV at 2×10^5 cells/mL in RPMI 1640 with 10% FCS and antibiotics. Cell counts determined by enzymatic dissociation at the times indicated. Viability was assessed by trypan blue dye exclusion. TGF β 1 was determined by EIA with monospecific kit from R & D Systems, Inc. The plateau phase of MIP-101 proliferation was associated with decreased production of TGF β 1.

Figure 5. Effect of HT-29KM conditioned medium on mucin expression. Medium conditioned by HT-29KM cells was added in varying proportions to fresh RPMI 1640 and 10% FCS from 0 - 50%. MIP-101 cells (2×10^4 per well) were added to individual wells of 96-well microtiter plates in RPMI 1640 with 10% FCS. After culture for 16 hr at 37°C, the medium was aspirated and 200 μ l of the conditioned medium or fresh medium added as indicated. Cells were then cultured for 5 days at 37°C in 5% CO₂. Triplicate wells were then tested for expression of mucin epitopes by EIA or for cell number by staining with crystal violet. Significant differences between B72.3 expression and MOPC controls are at least P<0.01 (*). All other mucin epitope expressions are similar to MOPC controls. The increase in B72.3 expression represents an increase in the expression of mucin per cell because the total number of cells decreased as the proportion of conditioned medium increased. This indicates that a factor(s) inhibited the proliferation of MIP-101 cells as the amount of B72.3 epitope increased.

Figure 6. Effect of EGF and TGF β 1 on mucin expression by MIP-101 cells. MIP-101 cells were incubated with the amounts of EGF or TGF β 1 (10 pg/mL) as indicated for 5 days in complete medium and then a cell-based EIA performed as described above (A). EGF stimulates B72.3 epitope expression but does not effect DF3 epitope at concentrations greater than 6 ng/mL. Anti-TGF β 1 monoclonal antibody was added to certain cultures in the presence of increasing amounts of EGF (B). Blockade of TGF β 1 by anti-TGF β 1 potentiates the effect of EGF on B72.3 epitope, but has no effect on the binding of DF3 or MOPC antibodies to MIP-101 cells.

Figure 1.

Figure 1A.

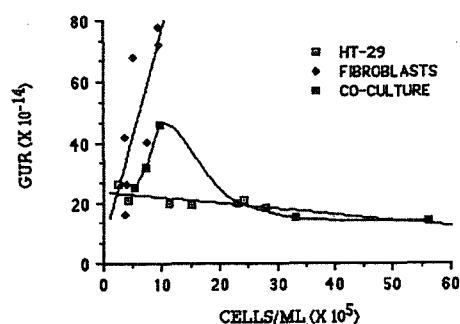


Figure 1B.

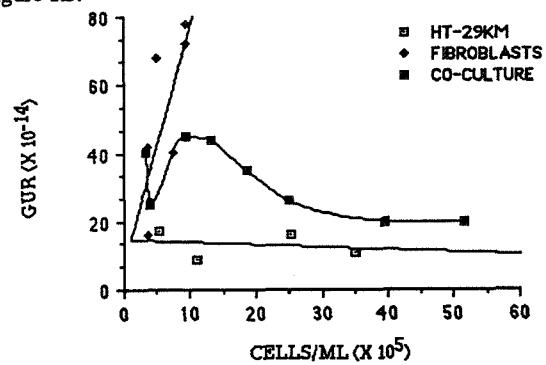


Figure 2. TGF β 1 Production in RWV and T25 Flask By CCL 188 Human Colorectal Carcinoma Cells

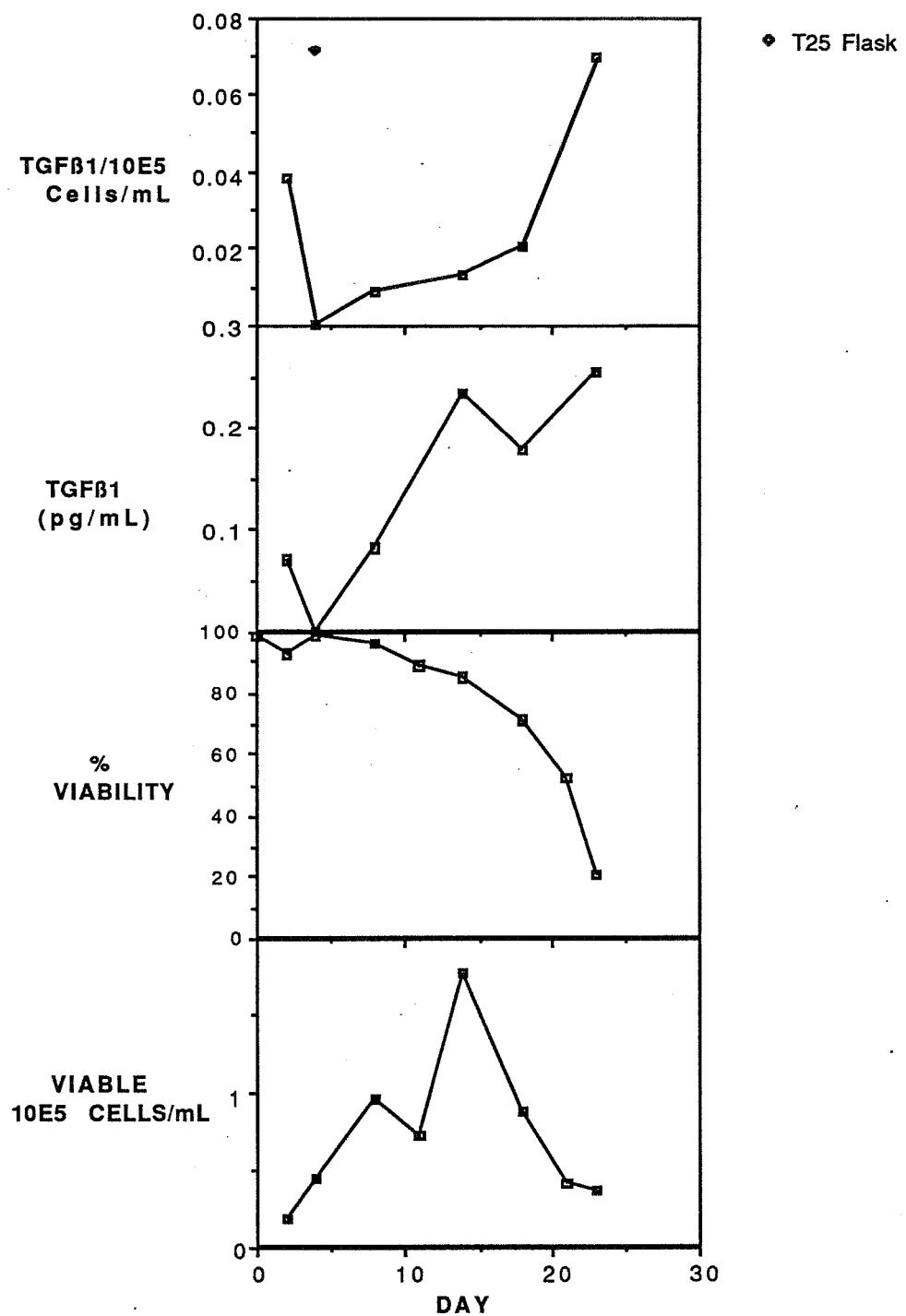


Figure 3.

PRODUCTION OF TGF β 1 BY KM-12c IN RWV AND T25 FLASKS

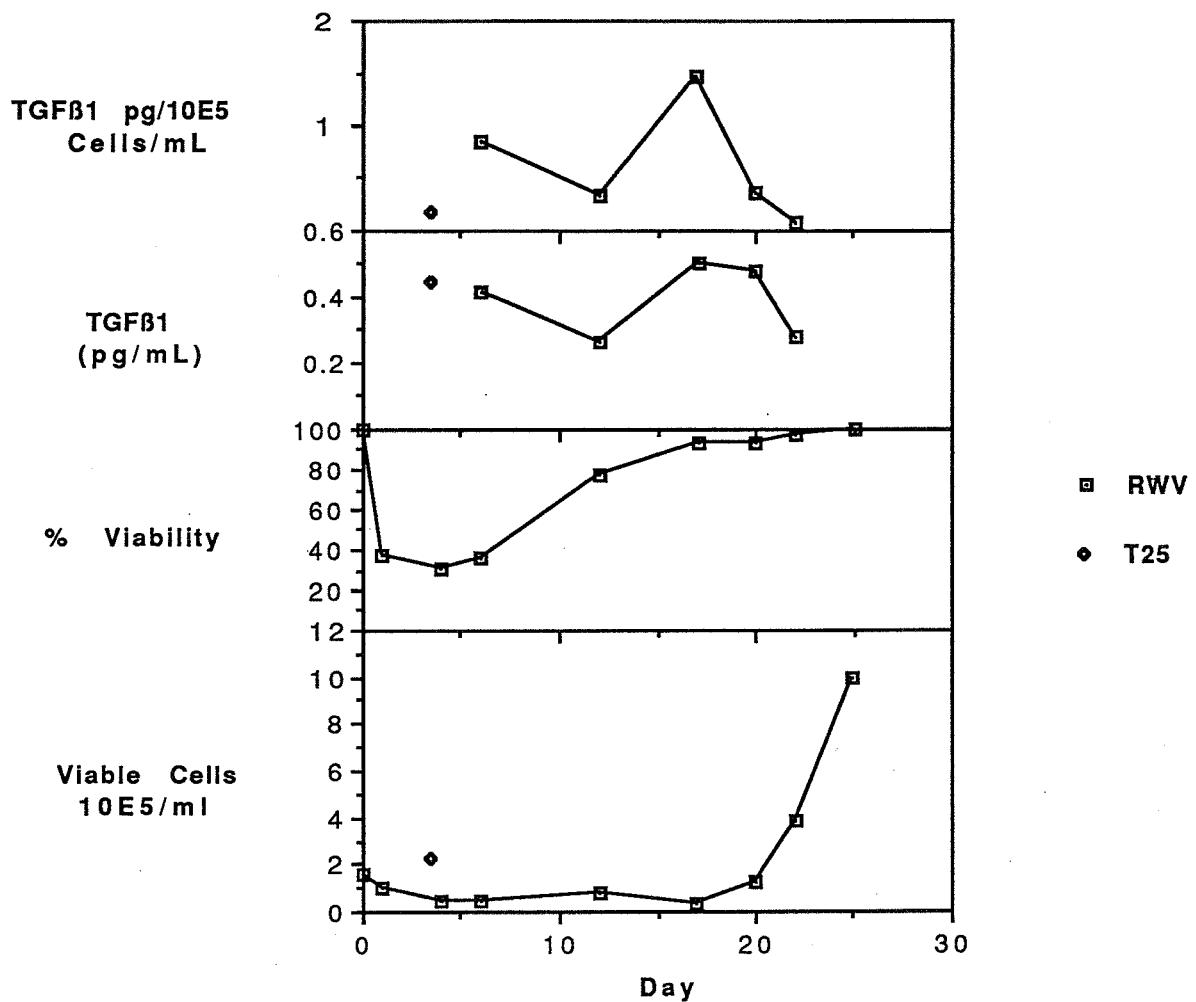


Figure 4.

TGF β 1 Production in RWV and T25 Flask By MIP-101 Cells

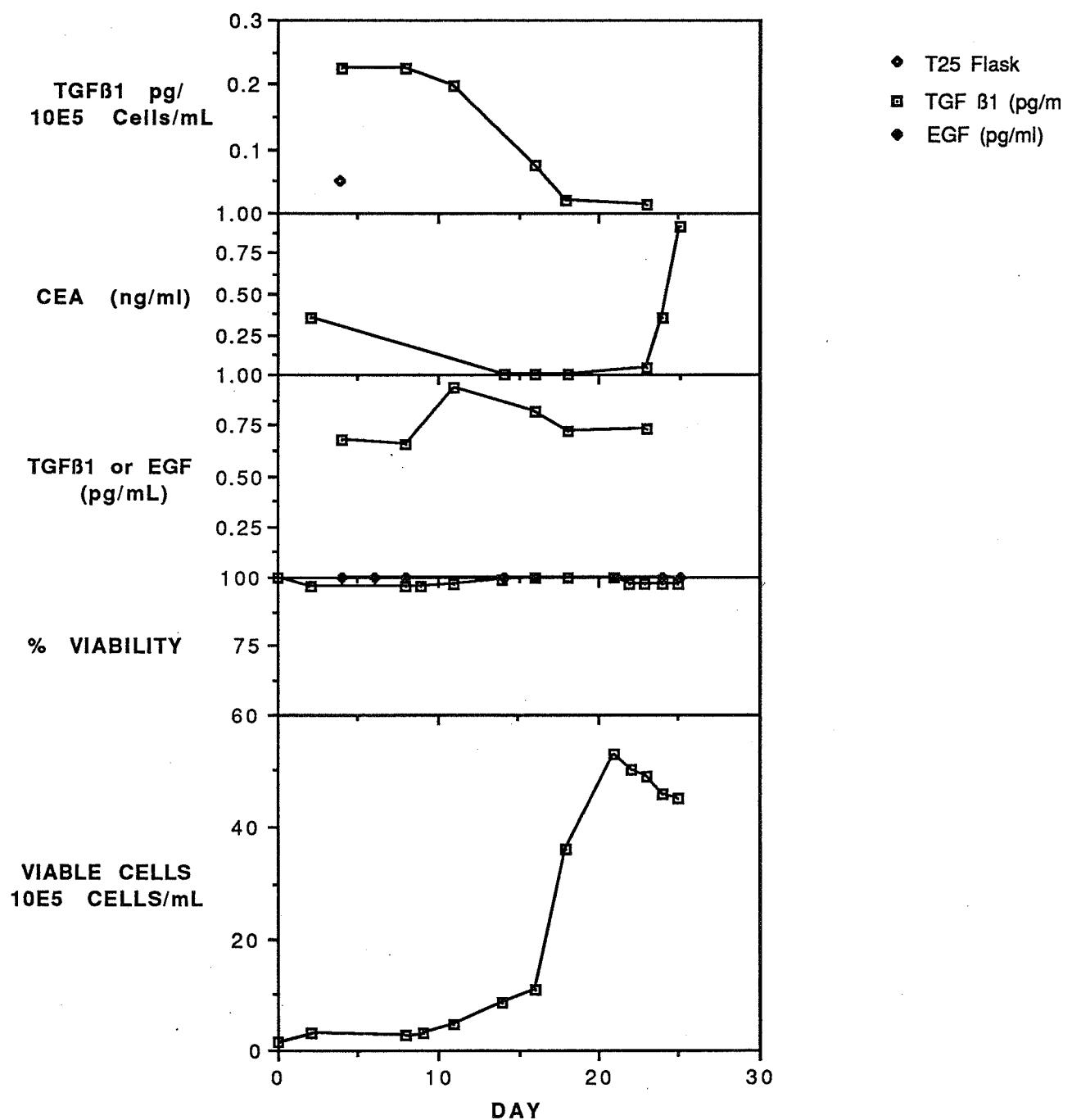
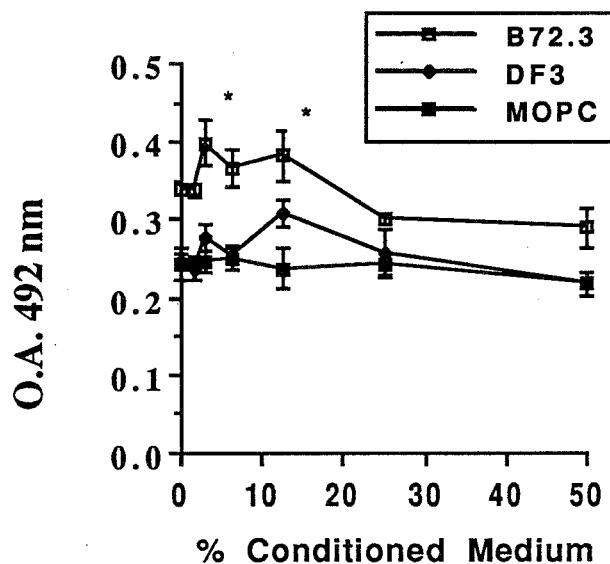


Figure 5.

Effect of Spent Medium on MIP-101 Mucin Expression



Number of MIP-101 Cells In Conditioned Media

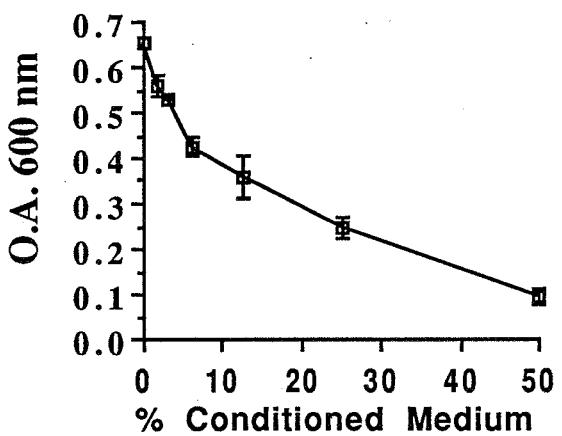


Figure 6A:

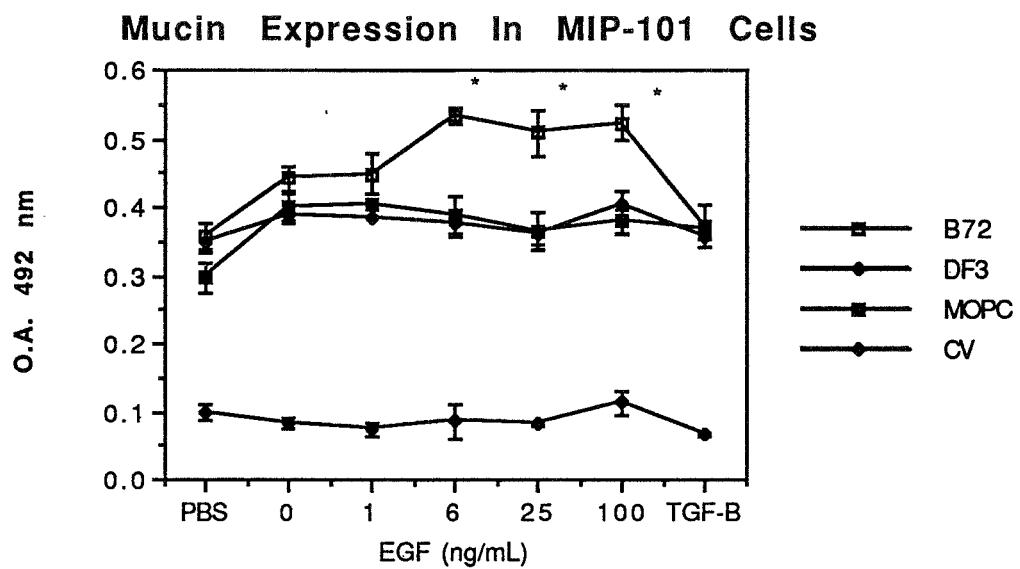
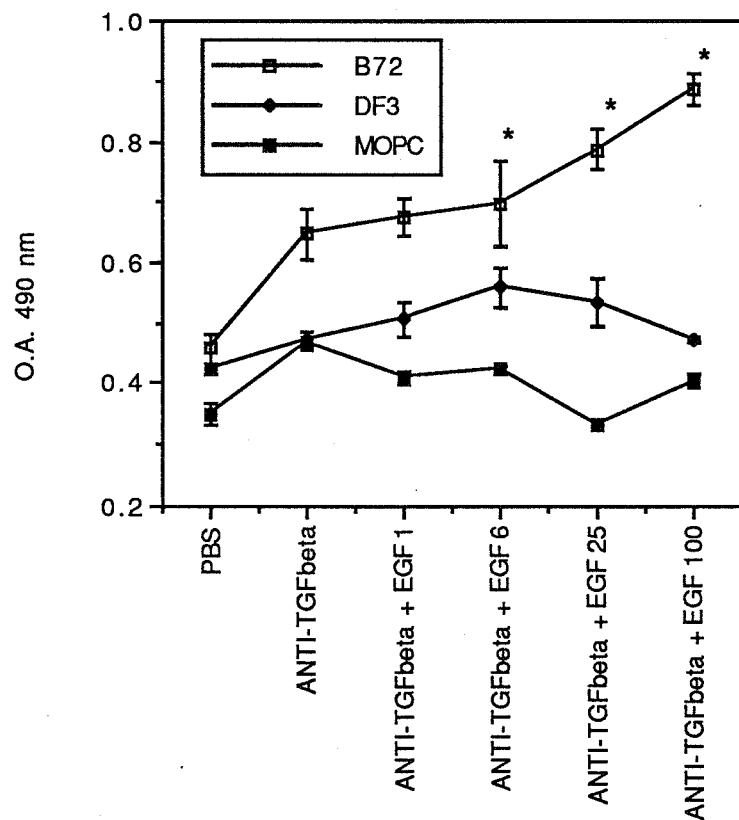


Figure 6B:



Prospects for Use of Microgravity-Based Bioreactors to Study Three-Dimensional Host-Tumor Interactions in Human Neoplasia

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Abstract Microgravity offers unique advantages for the cultivation of mammalian tissues because the lack of gravity-induced sedimentation supports three-dimensional growth in batch culture in aqueous medium. Bioreactors that simulate microgravity but operate in unit gravity provide conditions that permit human epithelial cells to grow to densities approaching 10^7 cells/ml on microcarriers in suspension, in masses up to 1 cm in diameter, and under conditions of low shear stress. While useful for many different applications in tissue culture, this culture system is especially useful for the analysis of the microenvironment in which host matrix and cells interact with infiltrating tumor cells. Growth in the microgravity-based bioreactor has supported morphological differentiation of human colon carcinoma cells when cultured with normal human stromal cells. Furthermore, these co-cultures produced factors that stimulated goblet cell production in normal colon cells in an *in vivo* bioassay. Early experiments also suggest that the microgravity environment will not alter the ability of epithelial cells to recognize and associate with each other and with constituents of basement membrane and extracellular matrix. These findings suggest that cells grown in bioreactors that simulate aspects of microgravity or under actual microgravity conditions will produce tissues and substances in sufficient quantity and at high enough concentration to promote characterization of molecules that control differentiation and neoplastic transformation. © 1993 Wiley-Liss, Inc.

Key words: microgravity-based bioreactors, three-dimensional host-tumor interactions, batch culture, epithelial cells, neoplastic transformation

Bioreactors have generally been used for the large scale production of microorganisms, cells, or their products. Biotechnology requires large quantities of cells to isolate molecules that are often in small concentration and not secreted into the medium. Current technologies for batch culture of cells have been well described elsewhere [Miller et al., 1989; Ramasubramanyan and Venkatasubramanian, 1990] and are beyond the scope of this review. However, a bioreactor that is based on principles derived from research in microgravity may be valuable in the research laboratory for the production of cells in three-dimensional structures that are large enough to facilitate molecular characterization. Microgravity-based bioreactors may be used to

probe aspects of cell biology that are difficult to define either in monolayer culture, embedded gel, or hollow fiber systems now available in the basic research laboratory. Microgravity-based bioreactors also may be suitable for large scale batch culture of mammalian cells. Our purpose in this review is to assess the use of a small-scale microgravity-based bioreactor operated in unit gravity on earth to analyze the interaction between human colon carcinoma cells and its host. We will describe the potential that such bioreactors may have for tissue formation when they operate in actual microgravity. The focus of our research is to characterize the molecular interactions between a neoplasm and its host to define molecules that may inhibit tumor growth. We will demonstrate how a microgravity-based bioreactor may aid in the analysis of complex tissue interactions with this problem as a paradigm for the cultivation of cells in a high fidelity three-dimensional architecture.

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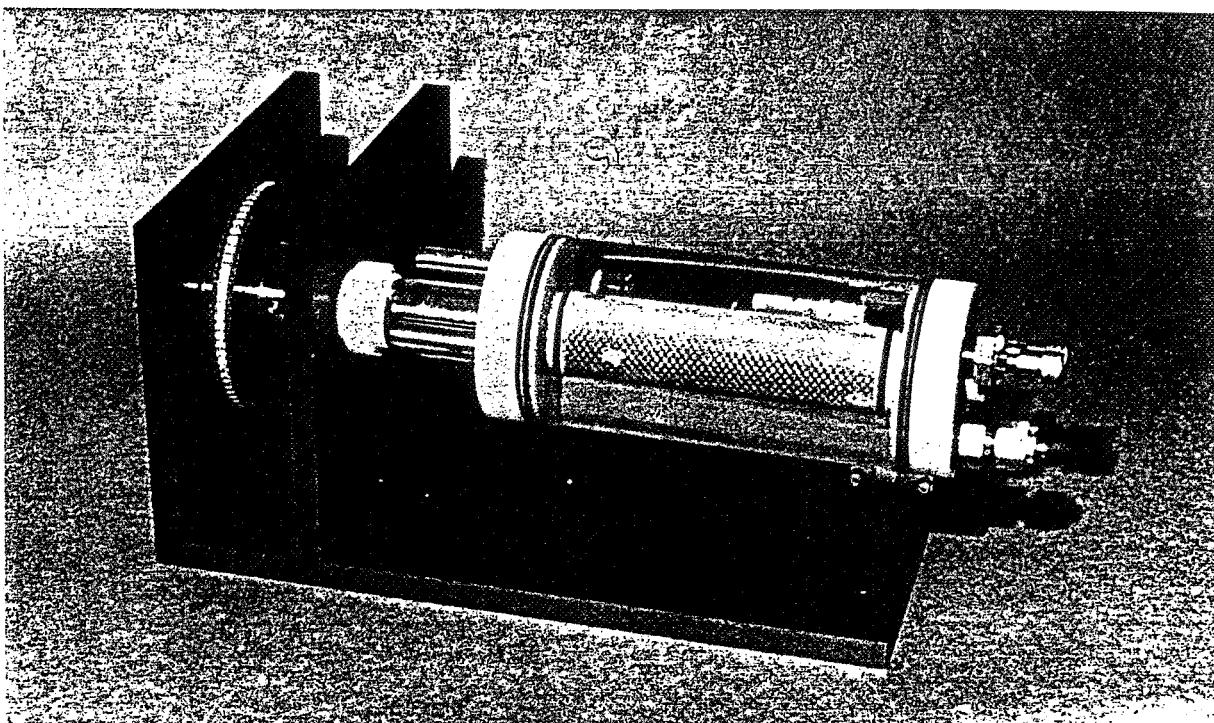


Fig. 1. The rotating wall vessel (RWV) is the microgravity-based bioreactor. Medium with microspheres is placed in the cylinder which is then rotated around the horizontal axis.

THE MICROGRAVITY-BASED BIOREACTOR

The microgravity-based bioreactor was developed at NASA-Johnson Space Center and is a zero-head space, aqueous medium-filled clinostat that suspends particles by rotating the vessel wall and spin filter (hence, the term rotating wall vessel or RWV) around the horizontal axis (Fig. 1). In its simplest form, the RWV has a capacity of 125–500 ml with a central spin filter covered with a semipermeable membrane that permits gas diffusion. Nonadherent cells are cultured in suspension, while adherent cells are grown on microcarrier beads. Cells or beads are maintained in suspension by balancing their sedimentation induced by gravity with centrifugation caused by vessel rotation. Rotation of the RWV at speeds of 10–60 rpm maintains particles that are up to 1 cm in laminar streamlines [Tsao et al., 1991], so that individual particles behave as though they were in a continuous fluidized bed reactor. This also means that cells or particles are subjected to a randomized gravity field with low shear stresses. Conventional bioreactor systems create a shear stress of 3–10 dyn/cm^2 [Cherry and Papoutsakis, 1986]. Since cell viability is decreased at shear stresses greater than 5–7 dyn/cm^2 [Cherry and Papoutsakis,

1986, 1988], most culture systems operate in a high shear environment that restricts growth. The RWVs provide a suspension culture environment that initially has low shear stress, since individual microcarrier beads (150–175- μm diameter collagen-coated dextran beads) are subjected to stresses of 0.81 dyn/cm^2 [Tsao et al., 1991]. However, when the microcarrier beads are covered with cells, cells and beads raft together and form dense cellular packs of 5–50 beads that are 0.7–1.5 cm in diameter. Vessel wall rotation must be continuously increased to counteract gravity-induced sedimentation. Eventually, as particles attain 1.0-cm diameter, the shear stress of gravity-induced sedimentation restrict further growth in unit gravity. Nonetheless, in unit gravity the microgravity-based bioreactor supports the cultivation on microcarriers of masses up to 1 cm in diameter and cell concentrations of at least 10^7 cells/ml.

ALTERNATIVES FOR THE STUDY OF COLONIC TISSUES IN VITRO

Several alternative culture systems are available in the research setting for the analysis of tumor-host interactions: static cell culture, static matrix cultures, roller bottles, stirred suspen-

TABLE I. Characteristics of Different Research Bioreactors

Reactor Vessel	Advantages	Disadvantages
Static cell culture	No shear stress, easy product isolation, clear morphologic analysis	Low cell density, only two-dimensional with interaction of 2 monolayers
Static matrix cultures	Three-dimensional cultures, no shear stress, permits analysis of morphology	Diffusion limited cell densities, product isolation difficult
Roller bottles	Relatively high cell density, product isolation easy, high shear stress	Two-dimensional with monolayers, can be used to support microcarriers but higher shear stress than microgravity-based bioreactor
Stirred suspension culture	Cell density of 10^6 - 10^7 cells/ml with microcarrier beads	Low-moderate shear stress, two-dimensional system
Airlift bioreactors	Cell density of 10^6 - 10^8 cells/ml depending on cell type, moderate shear stress	Cells are encapsulated, making cell recovery difficult
Hollow fiber-perfused system	High cell density of 10^7 - 10^8 cells/ml, easy product recovery, three-dimensional growth feasible, no shear stress on cells	Diffusion limited along length of fiber and through membrane into cell mass, recovery of cells may be very difficult
Microgravity-based bioreactor RWV	Low shear stress, high cell density (at least 10^7 cells/ml), three-dimensional cultures, easy product isolation	Cell culture limited to masses of 1 cm in unit gravity

sion culture, airlift bioreactors, and hollow fiber perfused cell systems are among the most commonly used systems (Table I). Each of these systems has advantages and disadvantages for the analysis of the host-tumor environment. Static cell cultures are the simplest culture systems, do not have any shear stress, allow careful morphologic analysis of the interaction of two monolayers of cells, and relatively easy isolation of products secreted into the environment. The disadvantage of the static cell culture system is that it is essentially two-dimensional because cells grow as monolayers on flat plastic or plastic-coated surfaces and the cell concentrations are limited to 0.3 - 1×10^6 cells/ml. Static matrix cultures such as those created with Matrigel [Kleinman et al., 1982] by Hall et al. [1982] are very useful for growing cells in a true three-dimensional architecture that permits morphologic analysis of cells. However, cell densities in a gel matrix culture are severely limited by diffusion of nutrients and wastes through the matrix. This limits the number of cells from which novel molecules may be isolated as well as hindering the isolation of substances that are secreted into the microenvironment. Roller bottle cultures are excellent for growing monolayers of cells or cells on microcarriers but have not been exploited for assessment of cell interactions. Stirred suspension cultures and airlift bioreactors allow for the growth of cells to cell concentrations of 10^7 - 10^8 cells/ml under conditions of fluid mixing that requires some shear

stress. These systems are usually used for the production of factors in batch cultures [Ray et al., 1990; Dean et al., 1987], and not used to study the interaction of different cell types. Finally, the most successful batch culture system available for the research laboratory is the perfused hollow fiber system in which cells are grown on one side of a semipermeable membrane while medium is perfused on the other side. This system has no shear stress and allows easy recovery of secreted products. The disadvantage is that diffusion of nutrients and wastes limits both the length of the fiber that can be perfused and the thickness of the cell mass that surrounds the fiber [Tharakan et al., 1988; Prokopenko and Rosenberg, 1989]. Nonetheless, the hollow fiber system allows for the three-dimensional culture of cells in the interstices between fibers [Rutzky et al., 1979].

The microgravity-based bioreactor compares favorably with these systems because it combines the favorable aspects of several of the other bioreactors. Since it uses microcarriers, it has a high surface area/volume ratio that facilitates nutrient and waste transfer. While the RWV is operated in a batch mode, secreted products may be isolated from spent medium more easily than can be done in matrix-based culture systems. While not as low as in a static system, the shear stress for particles that are less than 1 cm in diameter is quite low (0.92 dyn/cm^2) and less than the shear stress experienced in conventional stirred reactors. Furthermore, by using

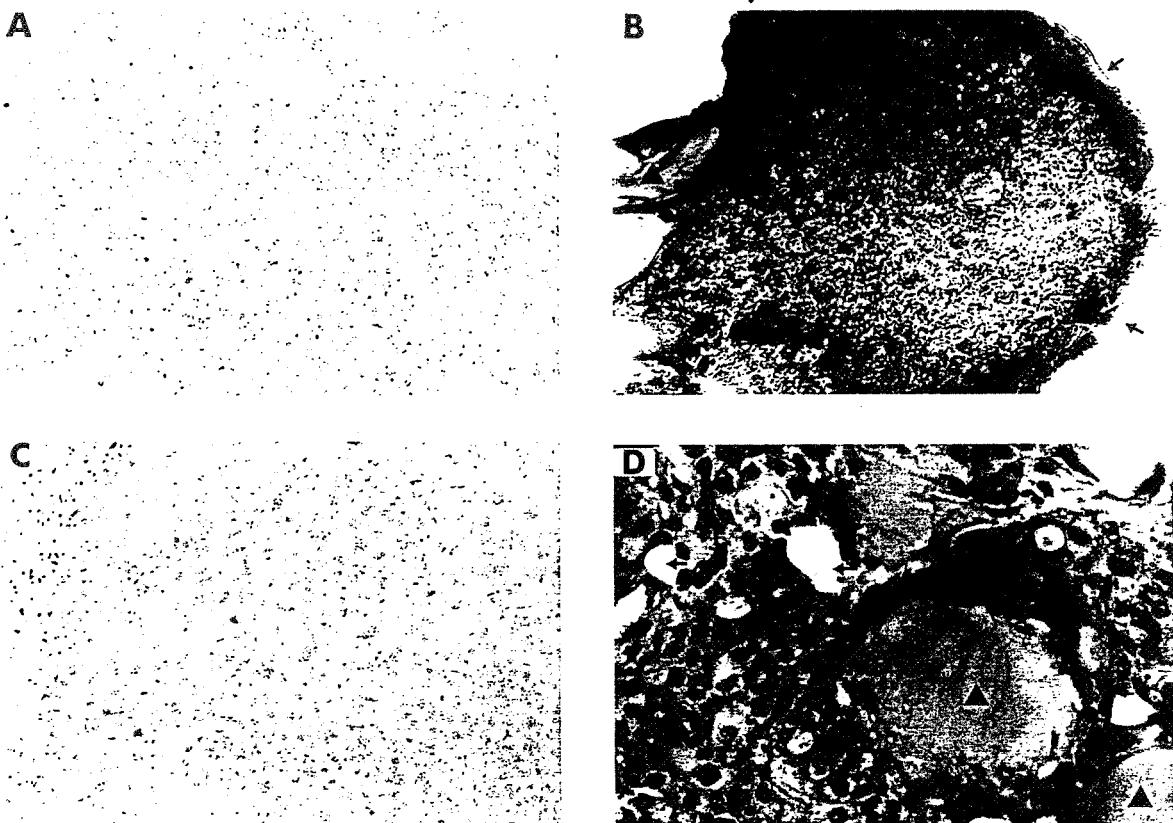


Fig. 2. Histologic comparison of mouse xenografts of HT-29 and HT-29KM vs. HT-29 and HT-29KM co-cultivated in RWVs; 5 μm H&E-stained cross sections of HT-29 (A) and HT-29KM (C) mouse xenografts, $\times 100$, HT-29 shows no differentiation and HT-29KM shows areas of limited cellular differentiation and signet ring cell development (lower left); 10 μm H&E stained co-cultures of HT-29(B) and HT-29KM(D), $\times 200$. B: A large polypoid growing out from the microcarrier (far left, triangle). Arrows mark the apex of the polypoid D, signet ring cell formations. D: In contrast to C, this tissue shows structural organization and differentiation. Multiple signet ring cells and glandular formation are visible. In addition, a line of columnar epithelial cells can be seen to the immediate left of the microcarrier in the center of the photograph (D). [From Goodwin et al., 1992.]

microcarriers, this type of bioreactor avoids encapsulating cells within calcium alginate beads that may prevent subsequent growth. The perfused hollow fiber system provides a good alternative to the RWV but the perfusion circuit may be more difficult to maintain than the simple batch operations in the RWV. The only disadvantage of the RWV system in its 125-ml configuration is that cells with stringent requirements must be seeded at a high enough concentration to sustain growth and this may require a total of $\sim 3 \times 10^7$ cells for cultures that require $2-3 \times 10^5$ cells/ml for initiation. Finally, while microcarrier systems are essentially two-dimensional spherical surfaces, the RWV allows for three-dimensional cultures since microcarrier beads may be rafted or aggregated by the cells growing on them. This permits anchorage-dependent cells to grow in three dimensions and in multiple

layers within the framework provided by several microbeads crosslinked together. Since the framework is open to aqueous medium, nutrient transfer is improved over that in hollow fiber systems where cells are severely limited in diffusion once there are more than three cell layers deposited on the hollow fiber membrane.

USES OF A MICROGRAVITY-BASED BIOREACTOR FOR HUMAN COLON CARCINOMA CULTURES

In our first evaluation of the RWV we compared the growth of HT-29, a human colon adenocarcinoma established as a cell line by Fogh [1975], and its subline HT-29KM in the bioreactor to implants of these two cell lines in athymic nude mice [Goodwin et al., 1992]. HT-29 is poorly differentiated both in nude mice (Fig. 2) and when grown alone in the bioreactor (Fig.

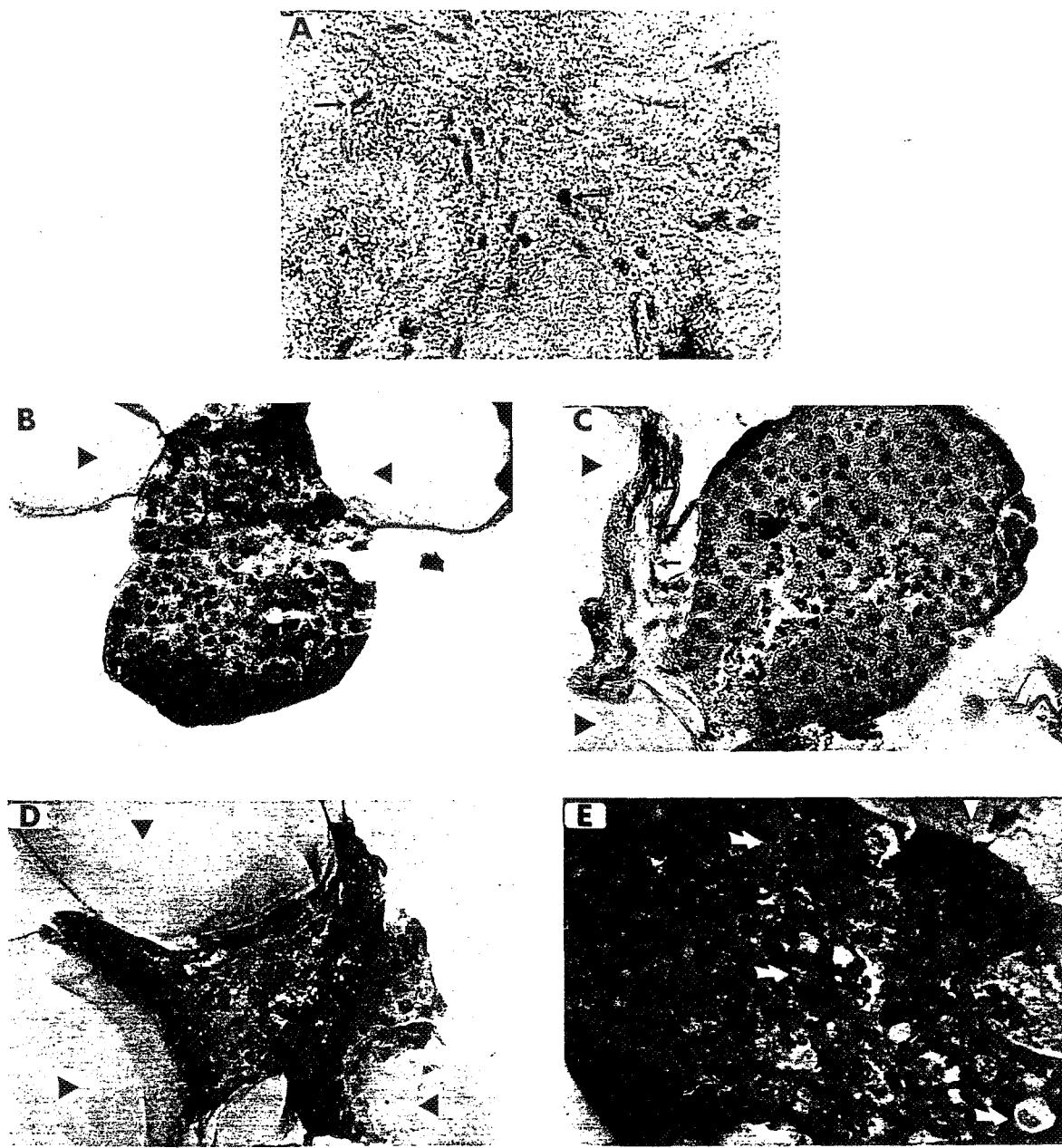


Fig. 3. Histologic comparison by mucicarmine of monocultured normal colon fibroblasts, HT-29 and HT-29KM vs. co-culture in RWVs; 10- μ m mucicarmine-stained cross sections of microcarrier bead packs (triangles) showing the normal colon fibroblasts (A), HT-29 (B), and HT-29KM (D) monoculture, $\times 200$; 10- μ m mucicarmine-stained cross sections of HT-29 (C) and HT-29KM (E) co-cultures $\times 200$. In contrast to the growth of HT-29 in monoculture (B), co-cultivation of HT-29 with normal colon fibroblasts results in polypoid structural development (C) which is similar to that seen *in vivo*. Note fibroblasts (arrows) on the surface of microcarriers (triangles). In contrast to the undifferentiated growth of HT-29KM (D), many packets of mucin (arrows) (differentiated secretory product), cellular differentiation, and organization can be seen in HT-29KM co-cultures (E). [From Goodwin et al., 1992.]

3) or co-cultured with human colonic fibroblasts in the bioreactor (Fig. 2). The subline HT-29KM is also poorly differentiated when cultured alone in the bioreactor but differentiates as a xenograft in nude mice producing glands and mucin-filled signet ring cells (Fig. 2). When co-cultured

in the bioreactor with human colonic fibroblasts, HT-29KM was induced to produce signet ring cells with mucin (Fig. 4) as well as glands (Fig. 2). Interestingly, when incubated with sodium butyrate or dimethyl sulfoxide, HT-29KM cells did not produce signet ring cells in stan-

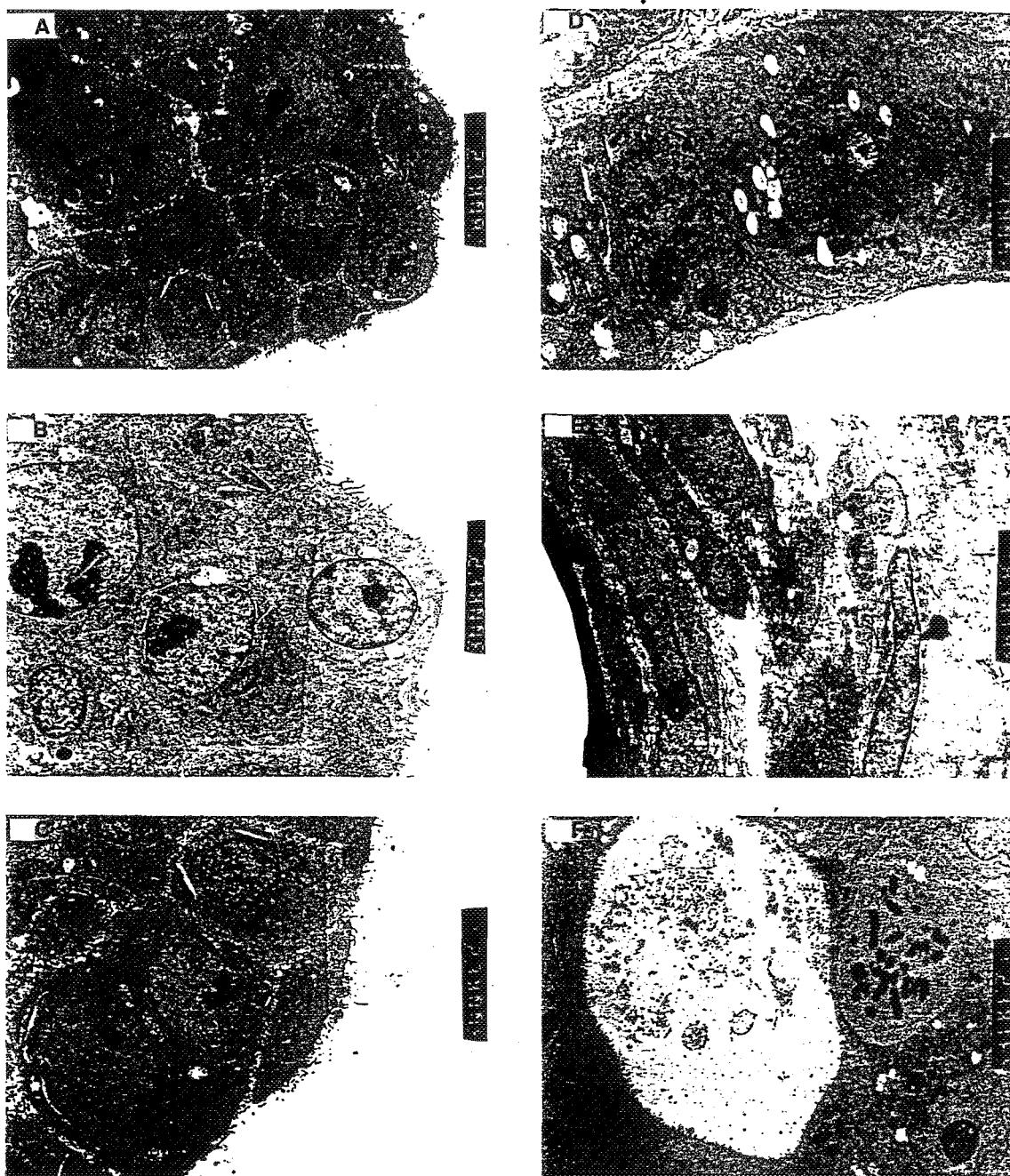


Fig. 4. TEMs of HT-29KM in RWV co-culture. HT-29KM co-cultivated with normal colon fibroblasts, $\times 5,400$ (A) and $\times 9,000$ (B,C). Note the regularity of the nuclei and reduction in multiple nucleoli, as compared to D and E. Note the presence of an apical microvillus border (A,C) and intercellular gland structures (F). HT-29KM co-cultured with normal colon fibroblasts, $\times 15,000$ (D), $\times 103,000$ (E), and $\times 7,000$ (F). Note the columnar epithelium, tight cellular junctions, and sinusoid formation in D. Normal colon fibroblasts layered over a microcarrier and large amounts of extracellular matrix produced in co-culture (E). F, interglandular structure formed by the junction of several cell borders with internal microvilli. [From Goodwin et al., 1992.]

dard monolayer cultures (data not shown). Attempts to induce mucin production in HT-29KM cells by glucose deprivation of confluent monolayers also failed in standard monolayer cultures. Since HT-29KM failed to differentiate

when cultured by itself in the RWV, the interaction of HT-29KM cells with human colonic fibroblasts in the co-culture induced the differentiation of the neoplastic cells. Kedinger et al. [1987, 1987a] have shown that the interaction of mes-

enchyme and epithelium is important for the formation of basement membranes and for the morphological state of differentiation of normal colonic epithelial cells. Richman and Bodmer [1988] demonstrated that three-dimensional cultures in appropriate substrates were most conducive to induction of differentiation and glandular formation by several human neoplastic colon cell lines. Richman and Bodmer [1988] also found that HT-29 did not form glands or produce signet ring cells when embedded in matrigel. Fukamachi et al. [1987] have observed that HT-29 cells do not differentiate when embedded in a fetal rat intestinal mesenchyme in a static gel matrix culture. Our data support the conclusion of these investigators. In addition, Bouziges et al. [1991] observed that HT-29 cells interacting with skin fibroblasts do not produce the matrix components necessary to form basement membranes. Similar observations were made in our system because human colonic fibroblasts did not secrete extracellular matrix when co-cultured with HT-29 but did when co-cultured with HT-29KM (Fig. 4). Since the colonic fibroblasts were harvested from similar pools of three primary lines established from donors who had had colonic resections for non-neoplastic disease, the induction of the extracellular matrix was stimulated by the neoplastic cells interacting with the same set of normal colonic fibroblasts. Further, there was evidence that the epithelial cells and fibroblasts established polarity with the fibroblasts close to the microcarrier surface and the epithelial cells further away and oriented in columnar arrays (Fig. 4). These results with HT-29 and its differentiable subline HT-29KM indicate that the microgravity-based bioreactor supports cell interactions that confirm findings observed in static gel matrix cultures as well as inducing structures that had previously only been observed in xenografts *in vivo*.

RWV CULTURE INDUCES CEA EXPRESSION IN A POORLY DIFFERENTIATED CARCINOMA

The induction of differentiation in three-dimensional cultures is demonstrated with the MIP-101 cell line that fails to differentiate in monolayer-based systems even in the presence of potent differentiation inducers. MIP-101 was established in culture from malignant cells harvested from the abdominal fluid of a patient with colon cancer that had disseminated to the liver, lungs, and peritoneum [Niles et al., 1987].

MIP-101 does not produce CEA in culture. It forms tumors in athymic nude mice when implanted in the subcutis of the flank, the spleen, or the abdominal cavity [Wagner et al., 1990]. MIP-101 produces carcinoembryonic antigen (CEA, a glycoprotein involved in intercellular adhesion that is a clinically useful tumor marker) when it grows in the abdominal cavity (its site of origin) but does not produce CEA when implanted in the spleen or the subcutaneous tissue. MIP-101 is weakly metastatic in nude mice when cells are taken from standard monolayer tissue cultures and injected intravenously or intrasplenically to produce experimental lung or liver metastases [Wagner et al., 1990]. It does not produce spontaneous metastases when implanted in the flank. However, when it is implanted in the abdominal cavity and produces CEA, it produces spontaneous metastases in the lungs or liver of athymic nude mice [Wagner et al., 1990]. Furthermore, nude mice that are pretreated with CEA intravenously followed by an intrasplenic injection of MIP-101 cells from standard tissue culture not only form experimental liver metastases but also experimental lung metastases [Wagner et al., 1990; Jessup, submitted].

These results suggest that (1) the production of CEA is important for MIP-101 cells to metastasize to other sites and (2) the production of CEA by MIP-101 cells is induced by the microenvironment of the abdominal cavity of the athymic nude mouse. In this view, the environments provided by the subcutaneous tissue or the spleen inhibit the production of CEA by MIP-101 cells, whereas some factor or condition in the abdominal cavity stimulates CEA expression. The induction of CEA expression then forms a test to analyze the effect of a three-dimensional environment upon MIP-101 cells. When MIP-101 cells are cultured in monolayer culture on various substrates (including Matrigel), CEA secretion was not stimulated (Table II). Even the incubation of MIP-101 cells with 2 mM sodium butyrate or DMSO for five days did not induce the production of CEA (Table II). No mRNA for CEA was identified in MIP-101 by polymerase chain reaction (data not shown). However, when MIP-101 cells were cultured in the RWV for five to seven days, they attained a concentration of $\sim 4 \times 10^6$ cells/ml and formed 3–4 mm spheroids that produced CEA (Table II). This induction of CEA expression in the RWV provides a new interpretation for the ef-

TABLE II. MIP-101 Cells Grown in the RWV Produce CEA^a

Treatment	Cells/ml ($\times 10^6$)	Type of culture	CEA (ng/ 10^6 cells/day)
None	0.13–1.30	Monolayer	0.0–0.95
Matrigel	0.13	Monolayer	0.5
Laminin	0.10	Monolayer	0.0
Butyrate (2 mM)	1.70	Monolayer	0.09
DMSO (1.25%)	0.12	Monolayer	0.38
RWV	0.58	RWV	76.79

^aCells were plated at 2×10^4 cells/ml either in T25 flasks or in 35-mm Petri dishes (on laminin or Matrigel coated surfaces) in RPMI 1640 medium with 10% fetal calf serum. The RWV cultures were initiated with 2×10^5 cells/ml. After 5 days, the medium was harvested and tested for the presence of CEA by RIA. The number of cells present was enumerated following enzymatic dissociation into a single cell suspension.

fect of the host microenvironment upon the induction of CEA expression in this neoplastic cell line. Prior to the RWV experiments the interpretation would be that the peritoneum, as the orthotopic site from which the cells were harvested, stimulated the cells to produce CEA and that the subcutaneous tissue or the spleen inhibited the expression of CEA. In light of the RWV results, the spleen and subcutaneous tissue may inhibit CEA expression but the abdominal cavity does not stimulate CEA production. Instead, the abdominal cavity permits CEA production when the cells grow as three-dimensional spheroids. Work is in progress to determine whether the induction of the expression of CEA in the RWV cultures is associated with an increase in the metastatic potential of MIP-101 cells in nude mice. Since the induction of CEA expression in this cell line is associated with aggressive behavior, it is also important to define how three-dimensional cultures stimulate CEA expression and whether the induction of CEA expression produces a more metastatic phenotype. The static matrix gel system involving Matrigel or other preparations of extracellular matrix may confirm that cells cultured as spheroids in three-dimensional cultures make CEA. However, access to a batch system such as the RWV that provides nearly 10^9 cells and > 100 ml of conditioned medium will greatly facilitate analysis of the molecules involved in the induction of CEA expression.

POTENTIAL FOR THE MICROGRAVITY-BASED BIOREACTOR TO PRODUCE GROWTH FACTORS

The HT-29 cell line is capable of differentiating into absorptive [Huet et al., 1987] or mucin-filled goblet cells [Phillips et al., 1988] under appropriate stimuli. These pathways of differentiation are similar to those taken by cells that arise from cycling stem cells at the base of the crypt of Lieberkuhn, migrate up the crypt as they terminally differentiate, and are released into the lumen of the gut. The process that controls the maturation and terminal differentiation of normal colonic epithelium is poorly understood but may involve soluble factors. Since human colorectal carcinomas implanted in athymic nude mouse bowel induce mucosal hypertrophy and hyperplasia over the implant [Hostetter et al., 1988; Sekikawa et al., 1990], we sought to determine whether substances released from human colorectal cells cultured *in vitro* induced similar changes in normal mouse colon. Spent medium from various types of tissue cultures was encapsulated in liposomes that were then implanted into the cecum of nude mice. Media from RWV cultures of HT-29 and HT-29KM induced not only a proliferation in the total number of cells in the crypts of Lieberkuhn but also a small, but significant expansion in the relative and absolute number of goblet cells (Table III). When media from monolayer co-cultures were tested in this bioassay, they did not stimulate the expansion of the goblet cell compartment and had a minor hyperplastic effect (data not shown). Similarly, roller bottle co-cultures also lacked this selective effect on goblet cell proliferation and maturation but did stimulate a potent hyperplastic response (Table III). While the identity of the factor stimulating production of goblet cells is unknown, it may be a new effect of previously defined growth factors or a novel intestinal growth factor similar to mucomodulin [Irimura et al., 1990]. Purified gastrin and epidermal growth factor while trophic, did not significantly increase the proportion of goblet cells (data not shown). Nonetheless, the RWV operating in unit gravity provides a culture system that may enhance the isolation of growth factors. A better understanding of the molecules that control the maturation and proliferation of normal colonic epithelial cells is important to ameliorating inflammatory bowel disease, the gastrointestinal effects of chemother-

TABLE III. Effect of Goblet Cell Differentiation Factor (GCDF) Produced in the RWVs on Murine Colon Crypt Cells

	Treatment	Crypt height ^a	Crypt cell no. ^b	Crypt goblet cell no. ^c	% Goblet cell ^d
Exp. 1 (RWV)	Saline	33.6 ± 0.9	36.3 ± 1.1	3.0 ± 0.2	10.7 ± 0.8
	Medium	32.2 ± 0.8	35.3 ± 0.9	3.8 ± 0.2	10.9 ± 0.6
	HT-29	44.8 ± 0.9*	42.5 ± 1.3	5.7 ± 0.3*	13.6 ± 0.8*
	Medium				
Exp. 2 (RWV)	Saline	58.5 ± 1.3	28.7 ± 0.7	6.0 ± 0.2	21.2 ± 0.9
	Medium	64.4 ± 1.4	33.4 ± 1.3	6.5 ± 0.3	19.5 ± 0.6
	Fibroblasts	56.9 ± 1.0	30.3 ± 0.7	5.6 ± 0.3	18.5 ± 1.1
	HT-29KM/ Fibro Coculture	90.9 ± 1.7*	33.0 ± 1.4*	9.8 ± 0.6*	25.6 ± 1.4*
Exp. 3 (Roller Bottle)	None	40.3 ± 1.0	20.2 ± 0.4	3.2 ± 0.2	15.8 ± 0.8
	Saline	66.2 ± 2.8	32.1 ± 1.5	5.1 ± 0.3	16.4 ± 1.1
	Medium	74.2 ± 2.1	33.1 ± 0.9	3.6 ± 0.2	11.0 ± 0.7
	HT-29	93.2 ± 2.3*	47.4 ± 1.0*	7.9 ± 0.3*	16.7 ± 0.7
	Medium				

*P < 0.01 compared to saline controls by ANOVA and Scheffé F test.

^aMean ± SEM of the height of sagittally sectioned crypts (in 1/250 mm) over submucosal liposomes.

^bCell number is the mean ± SEM of the total number of cells in one-half of a sagittally sectioned crypt.

^cGoblet Cell Number is the mean ± SEM of the number of goblet cells per half crypt.

^dPercentage of Goblet Cells is the goblet cell number divided by the number of cells per half crypt for each individual crypt (Mean ± SEM).

0.15M phosphate-buffered saline (saline), tissue culture medium (alpha MEM with 10% fetal calf serum, supplemented with 2 ng/ml of EGF) or culture conditioned by the HT-29 colon carcinoma cell line cultured in the RWV was encapsulated into liposomes; 0.05 ml of 6 µM of liposomes in PBS was injected into the submucosa BALB/c mice. The culture medium and supernatants had been concentrated 10-fold and one ml incorporated into 60 µM of CGP 19,835 A lipids manufactured by Ciba-Geigy. Five days later, the mice were killed and the colons harvested, irrigated with saline, and each cecum fixed in formalin. Paraffin sections were then stained with hematoxylin and eosin and the morphometric analysis was performed. Forty crypts were counted per treatment group that was sagitally sectioned over the liposomes. In Exps. 1 and 2, the medium was harvested from cultures in the RWV; in Exp. 3, the conditioned medium was collected from a roller bottle culture of HT-29. Particles in the roller bottle were less than 0.5 cm, while the RWV cultures contained many particles between 0.5 and 1.0 cm. While all the media stimulated colonic crypt hypertrophy (increased crypt height) and hyperplasia (increased crypt cell number), only the RWV cultures in Exps. 1 and 2 significantly expanded the percentage of goblet cells in the crypt. Thus, only the RWVs allowed for production of a detectable amount of a goblet cell stimulating activity.

apy, as well as providing a potentially new form of therapy for cancer.

MICROGRAVITY-BASED BIOREACTOR DOES NOT ALTER EPITHELIAL CELL ADHESION

If cells form masses in three-dimensions that accurately replicate either the structure or the function of tissue, then it may be possible that tissues can be engineered in microgravity. As described elsewhere in these Prospects, cartilage cells form cartilage on preformed biodegradable supports. This means that cartilage and, perhaps bone, can be grown to shapes designed by a computer to replace or supplant bone or cartilage that is missing either through a birth defect (e.g., craniofacial abnormalities) or through trauma. The design of more complex tissues

such as organs will be infinitely more complicated. However, one of the first steps in the formation of tissue is for the individual cells to recognize each other and then associate appropriately. It is not clear that in microgravity cells will be able to adhere to either extra- or intercellular adhesion ligands. We started analysis of this problem by assessing whether cells cultured in the RWV for 5 or more days are capable of binding to standard extracellular matrix proteins such as laminin and fibronectin as well as the intercellular adhesion glycoprotein CEA. Cells cultured in simulated microgravity have the same capability of binding to adhesion ligands as do cells grown in standard monolayer cultures (Table IV). This suggests that cells will maintain their ability to sort and form struc-

TABLE IV. Culture in the RWV Does Not Inhibit the Ability of Epithelial Cells to Bind to Inter- and Extracellular Ligands^a

Substrate to which cells adhere	% Cells bound (Mean ± SEM)					
	KM-12c		MIP-101		CCL 188	
	RWV	Control	RWV	Control	RWV	Control
None	6 ± 1	3 ± .1	14 ± 1	10 ± 1	20 ± 2	1 ± .1
CEA	32 ± 3*	6 ± 0.3*	13 ± 1	9 ± 1	31 ± 3*	16 ± 1*
Collagen IV	58 ± 2	26 ± 1*	29 ± 1*	61 ± 2*	47 ± 4*	54 ± 0.6*
Fibronectin	3 ± 1	4 ± 1	11 ± 1	11 ± .3	22 ± 2	1 ± 0.1
Laminin	57 ± 9*	19 ± 0.4*	53 ± 1*	63 ± 2*	25 ± 2	43 ± 4*

^aCells were started from the same stock and harvested from standard monolayer or RWV cultures after 6–7 days and analyzed for their ability to adhere to various ligands.

*Significant differences between the control (None) group and experimental groups of at least $P < 0.01$ by Scheffé test. While the magnitude of the adhesion to the various substrates may change with different culture conditions, the ability to bind is not impaired in the RWV culture. This suggests that cells will be able to associate in actual microgravity as well as they do in unit gravity.

tures in actual microgravity conditions and that microgravity may permit the creation of novel tissues.

POTENTIAL FOR CULTURE SYSTEMS OPERATING IN MICROGRAVITY

The microgravity-based bioreactor was designed to simulate certain aspects of microgravity. It was also designed to operate in a microgravity environment under process control. As of this writing, the bioreactor has flown short shuttle missions to determine whether the microcarriers will stay suspended as predicted when vessel wall rotation is stopped in microgravity. Confirmation of the lack of sedimentation in aqueous medium has recently been achieved for microcarriers in microgravity. This observation means that tissue cultures may be able to routinely form and replicate delicate three-dimensional structures. Should three-dimensional structures like the crypts of Lieberkuhn that line the mucosa of the large bowel be maintained in batch culture, the molecules that regulate proliferation and differentiation within these structures may be more easily identified and applied to situations in unit gravity. The characterization of such factors would be of tremendous medical and biologic benefit.

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ABSTRACT ONE

USE OF MICROGRAVITY EMULATION TO TEST THE FUNCTION OF THREE-DIMENSIONAL HUMAN COLON CARCINOMA CELL CULTURES. J.M.Jessup, R. Ford, G. Spaulding, and T.J Goodwin. New England Deaconess Hospital, Boston, MA and NASA-JSC, Houston Texas.

Carcinoembryonic Antigen (CEA) is an oncofetal protein produced by many human carcinomas involved in intercellular recognition and adhesion, and important to metastasis or the spreading of tumor to distant organs. The purpose of this study was to determine whether human colon carcinoma cells (MIP-101) produce CEA in a rotating wall vessel (RWV) designed by NASA/JSC. The RWV emulates microgravity for tissue culture by permitting cells to grow in three-dimensional matrices under low shear stress in aqueous medium. Most in vitro culture systems for mammalian cells are two-dimensional. However, cells exist in a three-dimensional tissue matrix in vivo and cellular interactions created within the matrix define differentiation and function of the tissue. MIP-101 cells implanted intraperitoneally produce CEA and form spontaneous liver and lung metastases; however, MIP-101 does not produce CEA either in monolayer culture in vitro or when placed in the sub cutis of nude mice where it produces tumors, but no liver or lung metastases. Systemic pretreatment of athymic nude mice with CEA increases the number of liver and lung colonies produced by MIP-101 cells injected intrasplenically. Thus different matrices have contrasting effects on CEA expression: the sub cutis inhibits and the peritoneum enhances CEA expression. To test the function of different substrates and the RWV on MIP-101 production of CEA, 2×10^5 MIP-101 cells/ml were seeded into T25 flasks in monolayer culture with differentiation inducers or basement membrane or matrix components or RWV and the induction of CEA expression measured by an ELISA in the medium. The production of CEA was then expressed on a cellular basis.

Treatment	Cells ($\times 10^6$)	Type of Culture	CEA (ng/ 10^6 Cells/day)
None	0.66 - 7.5	Monolayer	0.0 - 0.95
Matrigel	0.66	Monolayer	0.50
Laminin	0.50	Monolayer	0.00
Butyrate (2mM)	8.50	Monolayer	0.09
DMSO (1.25%)	0.60	Monolayer	0.38
RWV	70.00	RWV	76.79

Although cultivation of MIP-101 cells on matrigel or laminin stimulated the appearance of spindle shaped cells, none of the inducing agents or substrates caused CEA expression. However, the RWV promotes the expression of CEA by MIP-101 cells. Further, the data from the RWV suggests that sub cutis matrix inhibits CEA expression while the peritoneal matrix supports it. These results are important because they permit a functional analysis of three-dimensional tissue interactions in a system that emulates aspects of microgravity.

Presented at COSPAR meeting in August 1992 in Washington, D.C.

ABSTRACT TWO

EFFECT OF SIMULATED MICROGRAVITY ON HUMAN EPITHELIAL CELI ASSOCIATION AND RECOGNITION. J.M. Jessup*, R. Ford, T.J. Goodwin, G. Spaulding Harvard Medical School, Boston, MA 02215 and NASA/Johnson Space Center, Houston, TX 77058.

INTRODUCTION. Cell differentiation in microgravity requires intercellular recognition and adhesion. We tested the hypothesis that simulated microgravity does not inhibit cell attachment to adhesion molecules. METHODS. Human colorectal carcinoma cells (5×10^5 MIP-101 or KM-12c cells/ml) were cultured in the NASA Rotating Wall Vessel (RWV). Cells in the RWV are under low shear stress with randomization of the gravity vector. Cells were harvested at 6 - 7 days, labelled with ^{51}Cr , and assayed for binding to carcinoembryonic antigen (CEA), collagen, laminin, or fibronectin in microtiter plates. Cells (5×10^4 /well) were incubated for 90 min at 37°C , nonadherent cells washed off and the adherent cells counted. Control cells were grown in standard tissue culture flasks. Differences between means assessed by ANOVA with Bold print $P < 0.01$ versus None control. RESULTS:

Substrate To Which Cells Adhere:	% CELLS BOUND (MEAN \pm SEM)			
	KM-12c		MIP-101	
	RWV	Control	RWV	Control
None (Plastic Alone)	6 \pm 1	3 \pm .1	14 \pm 1	10 \pm 1
CEA	32 \pm 3	6 \pm .3	13 \pm 1	9 \pm 1
Collagen IV	58 \pm 2	26 \pm 1	29 \pm 1	61 \pm 2
Fibronectin	3 \pm 1	4 \pm 1	11 \pm 1	11 \pm .3
Laminin	57 \pm 16	19 \pm .4	53 \pm 1	63 \pm 2

The RWV cultures give similar results to the Control cultures for binding to basement membrane proteins and to CEA, an epithelial intercellular recognition molecule. Thus, microgravity is not likely to alter cell association and adhesion.

Presented at the national meeting of the Aerospace Medical Association in Miami, Florida in May 1992.

ABSTRACT THREE

CEA PRODUCTION IN HUMAN COLON CANCER J. MILBURN JESSUP, W. Fitzgerald, R Ford, J. Polanec, and D. Brown. Deaconess Hospital, Boston, MA. 02215 and *Krug Life Sciences NASA/Johnson Space Center, Houston, TX 77058.

Malignant cells must implant, invade, and proliferate within an organ to create a metastasis. Recently, we have found that Carcinoembryonic Antigen (CEA), a 180 kD glycoprotein of the Ig supergene family, promotes colonization of nude mouse liver and lung when injected intravenously into nude mice prior to intrasplenic injection of 2×10^6 poorly differentiated, human MIP-101 colon carcinoma cells. MIP-101 cells grown in monolayer culture or in the flanks of nude produce tumors without either CEA or Metastases. MIP-101 cells implanted in the peritoneum produce local tumors CEA, and liver and lung metastases. We tested whether the 3-D culture system of the NASA Rotating Wall Vessel (RWV) would support CEA production when MIP-101 cells were cultured on microcarrier beads. MIP-101 cells grew to densities of $\sim 6 \times 10^6$ cells/ml from $1-3 \times 10^5$ cells/ml over 7 -10 days in GTSF or RPMI 1640 media with 10% FCS. MIP-101 cells produced low quantities of CEA (0.5 -1.0 ng/ml) during the plateau phase of cultures. However, viability decreased once cells were cultured past 9 days associated with medium pH of 6.5 - 6.8. Glucose utilization decreased and cells came off microcarrier beads. O₂ tension was > 80 mm Hg but PCO₂ was 80 - 120 mm Hg. MIP-101 cells from the RWV were tumorigenic but no more metastatic than monolayer culture cells. We conclude that 1) the RWV induces high metabolic activity in MIP-101 cells, 2) as culture senesces CEA is released into the medium, and 3) better pH control is needed.

Presented at the national meeting of the Tissue Culture Association in Raleigh-Durham, NC in June 1994.

ABSTRACT FOUR

GROWTH OF HUMAN COLORECTAL CARCINOMA CELLS IN NASA ROTATING WALL VESSEL (RWV) PRODUCES HIGH CELL YIELDS WITH DIFFERENTIATION AND REDUCED GLUCOSE CONSUMPTION. J.M. Jessup, T.J. Goodwin, D.A. Wolf, G. Spaulding. Deaconess Hospital, Boston, MA and NASA-Johnson Space Center, Houston, TX.

Successful batch culture of mammalian cells requires efficient mass transfer of nutrients and wastes in an environment that attains high cell concentrations. The NASA-JSC RWV Bioreactor has several ideal characteristics for such batch cultures since it is a zero headspace reactor that rotates around the horizontal axis and produces low shear stress (<3 dynes/cm) for microcarrier bead-based cultures. Gas exchanges through a central silicone-covered spindle. Cultures are not exposed to a gas-liquid interface with attendant surface denaturation effects. A poorly differentiated human colon carcinoma cell line, HT-29, and a subline, HT-29KM, that differentiates when grown in nude mice were cultured in the RWV and in 5 ml T25 conventional monolayer cultures for periods of up to 30 days. Maximum concentrations of both lines in conventional cultures were 3×10^5 cells/ml in T25 flasks, 3×10^6 cells/ml in the RWV, and 9×10^6 cells/ml in the RWV when co-cultured with human colonic fibroblasts. Both lines were poorly differentiated in T25 and RWV monocultures and in T25 co-cultures with human colon fibroblasts. Co-cultures of HT-29 with human colon fibroblasts did not differentiate in the RWV but co-cultures of HT-29KM formed glands and mucin-filled signet ring cells. HT-29 and HT-29KM co-cultures produced cell cycle lengthening associated with a decrease in glucose consumption from 47×10^{-14} M/hr/cell at 9 days of culture to 9×10^{-14} M/hr/cell at 25 days of culture with viabilities of > 95% without LDH or SGOT released into medium. Monocultures of fibroblasts did not display a decrease in glucose consumption in the RWV vs T25 flasks but HT-29KM and HT-29 in the RWV were 16 - 30% of T25 flask consumption. These results demonstrate that 1) zero head space bioreactors provide an environment in which cells may recreate architectures that are similar to *in vivo* tissue but not in conventional *in vitro* systems, 2) high density, viable RWV cultures display reductions in glucose consumption that suggest epithelial cells alter their energy utilization in the RWV.

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